INTERFERENCE MECHANISMS OF ATTACHING/EFFACING PATHOGENS WITH THE HOST INFLAMMATORY RESPONSE

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Abstract

Attaching and effacing (A/E) pathogens contribute to a significant burden of diarrheal disease, which accounts for 10% of childhood deaths under age 5 worldwide. These Gram-negative bacterial pathogens have evolved sophisticated and complex mechanism to regulate the host immune responses and manipulate their microenvironment. Through combined approaches including *in vivo* *Citrobacter rodentium* mouse infection models and *in vitro* protein assays, we have uncovered a novel mechanism by which A/E pathogens regulate the nuclear factor kappa B (NF-κB) signaling pathway in the host cell. Briefly, A/E pathogens encode a type III secretion system that is used to deliver virulence proteins (effectors) into the host cell. Among these, the zinc metalloprotease, NleC, targets p65 within the host cell as a substrate for cleavage. While only cleaving a small percentage of the total p65 molecules within the cell, NleC mediates a robust effect on inflammatory signaling. This amplification is achieved by the interaction between the small N-terminal fragment of p65, produced by NleC cleavage, with RPS3, the NF-κB promoter specifier. RPS3 is a non-Rel subunit protein present within the NF-κB complex that targets the transcriptional activation of a select sub-set of NF-κB target genes. Thus, we have revealed a novel mechanism by which A/E pathogens regulate gene expression within the host that may serve as a model for the many other pathogen-encoded proteases that selectively target p65 as a substrate. We have also identified a novel virulence factor in *C. rodentium* that abrogates the pathogen's ability to generate an inflammatory response following infection. Disruption of a DNA recombinase prevents the intimate attachment of *C. rodentium* to colon epithelial cells in the IL-22$^{-/-}$ mouse which is exquisitely permissive to infection, however further work is need to determine the mechanism by which this occurs. The work presented in this thesis serves as an example of the importance of examining how the host-pathogen interactions observed during infection alter the transcriptional regulation that occurs on each side of the battle.

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1 Introduction

1.1 Attaching and effacing pathogens

Diarrheal disease is responsible for approximately 10% of the deaths in children under 5 worldwide, with the majority of that burden being shouldered by the developing world (Croxen et al., 2013; Kotloff et al., 2013; Liu et al., 2012). Rotavirus, *Cryptosporidium*, and pathogenic bacterial infections are the leading causes of diarrheal disease with the Gram-negative *Escherichia coli* (*E. coli*) pathogens accounting for a large burden of those cases (Croxen et al., 2013). One type of pathogenic *E. coli* strains are classified as attaching and effacing (A/E) pathogens due to the characteristic lesion formed on the epithelial brush border upon infection and are transmitted through the fecal-oral route. The characteristic A/E lesion occurs by the delivery of the bacterial receptor Tir into the host cell, via a type three-secretion system (T3SS) (McDaniel et al., 1995), causing the rearrangement of actin and many other host cellular proteins to form pedestals (Goosney et al., 2000). It is through these formations the bacteria are able to anchor themselves onto the epithelial cell and cause disease, however the specific mechanisms by which these pathogens cause disease remains an area of active investigation.

1.1.1 EPEC/EHEC

There are six currently recognized pathogenic profiles of *E. coli* that range in their expression of virulence factors, infection sites, and disease profile. Of these, enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) are among the best studied. EPEC is a leading cause of diarrhea in infants, 0-11 months in age, in low-income countries (Croxen et al., 2013; Kotloff et al., 2013). Estimates of mortality following dehydration range as high as 1 million infants a year (Mead et al., 1999). EPEC was first isolated following a series of diarrheal outbreaks among infants in the 1940s and 1950s (Robins-Browne, 1987). Since then, the disease outbreaks associated with EPEC

have shifted from primarily in the developed world into the developing world, with sporadic food/water borne outbreaks still occurring within the developed world (Croxen et al., 2013).

EHEC, however, is more prevalent in developed countries and differentiates itself mainly through the expression of Shiga toxin 1 or 2 (stx1 or stx2). The most commonly isolated serogroup of EHEC, O157:H7, is associated with contaminated food and water borne exposure. EHEC causes a more severe form of diarrhea that, in some, is accompanied by hemorrhagic colitis resulting in hospitalizations in 42% of cases and sometimes death (Croxen et al., 2013; Levine, 1987). In approximately 15% of childhood infections, EHEC causes hemolytic uremic syndrome, leading to acute renal failure (Law et al., 2013; Tarr et al., 2005). The severity of disease caused by EHEC is attributed to toxin production and release. While the regulatory mechanisms of *Stx* gene expression are yet unclear, hydrogen peroxide exposure from neutrophils in the gut and antibiotic treatment are known to facilitate the release of stx toxins into the gut (Johannes and Romer, 2010). The toxin blocks protein synthesis by selectively targeting the 3' end of 28S rRNA, which results in deficient host cellular processes and inflammatory signaling.

EPEC and EHEC have a limited range of hosts for infection making studying these infections *in vivo* extremely challenging. The restricted host range has necessitated the use of related bacterial strains for the development of animal models, which has given rise to the *C. rodentium* – murine infection model.

1.1.2 C. rodentium

The rodent-restricted pathogen, non-motile, *Citrobacter rodentium* (*C. rodentium*), was isolated following a series of disease outbreaks in mouse colonies and was originally classified as a murine pathogenic *E. coli* (Petty et al., 2010). However, it was determined that *C. rodentium* caused this transmissible colonic hyperplasia resulting in colonic thickening that could spread to co-housed mice (Mundy et al., 2005). Following its isolation, *C. rodentium* was used to examine host pathogen

interaction within the gut, providing a robust animal model for the study of A/E pathogens. Prior to the use of the *C. rodentium*-mouse infection model, studies on A/E pathogens were limited to *in vitro* experimental systems where characterizing the immune response to infection was restricted to innate signaling pathways.

In orally inoculated mice, the non-invasive *C. rodentium* colonizes the cecum within a few hours and within a few days spreads to the distal colon (Wiles et al., 2004). This is followed by the infiltration of inflammatory cells, mostly neutrophils. Bacterial numbers reach their peak around 14 days post infection, and complete clearance is achieved by 28 days post infection (Wiles et al., 2004). The immune response to *C. rodentium* infection elicits a robust CD4 T cell response and antibody response that provides sterilizing immunity to subsequent infections (Collins et al., 2014). Despite *C. rodentium* being a natural mouse pathogen there are variations in the susceptibility of each strain to morbidity and mortality, with C57Bl/6 mice experiencing limited mortality while C3H/HeJ mice experiencing high mortality (Mundy et al., 2005). Infection in mice recapitulates many of the same clinical manifestations of EPEC and EHEC infection in humans and therefore *C. rodentium* became widely used as a model to study the innate and adaptive immune response to A/E infection (Collins et al., 2014; Koroleva et al., 2015; Lebeis et al., 2008; Mundy et al., 2005). This is particularly important since neither EPEC nor EHEC can naturally infect mice, making understanding these pathogens challenging. EPEC, EHEC, and *C. rodentium* share 67% of their genes (Petty et al., 2010), among which are the LEE pathogenicity island, the T3SS, and many of the effectors used to modulate their microenvironment (Collins et al., 2014). The interactions of these effectors with many host proteins has led to the further characterization of host responses toward A/E pathogens, providing additional insight into the complex responses following infection with EPEC or EHEC. However, novel effector proteins continue to be identified and characterized for their roles in mediating pathogenesis. As the list of effectors and their functions grow, so does the appreciation of the interaction with host signaling cascades, which remains a fertile area of study.

Aside from acting as a model for studying infection, the inflammatory response following *C. rodentium* colonization has been used to model Crohn's disease and ulcerative colitis. The involvement of bacterial immune stimulation and the differences in microbiota between healthy controls and IBD patients suggest that bacteria play an important role in driving IBD (Wu and Wu, 2012). The role of the microbiota has been widely studied for its interaction with the immune sensors in the gut. The interplay between the innate immune system and the microbiota commonly induce immune tolerance, where the immune system will not react to commensal organisms but still activate in response to colonization by foreign pathogens (Wu and Wu, 2012). Studies using germ free mice have demonstrated that the presence of the microbiota can modulate the responses of immune cells and lead to skewed immune responses (Bergstrom et al., 2012; Buffie and Pamer, 2013; Collins et al., 2014; Lievin-Le Moal and Servin, 2006; Wu and Wu, 2012). It appears that antigens from the microbiota prime the innate and adaptive responses by stimulating specific responses, leading to constitutive low level mucus production, antimicrobial peptides and appropriate T cell responses, which work to keep the microbiota in check (reviewed in (Lievin-Le Moal and Servin, 2006; Wu and Wu, 2012)). These immune stimulatory effects and the nutrient-competitive environment of the gut also provide another level of protection against pathogen invasion. In fact, when the microbiota of mouse strains traditionally resistant to infection by *C. rodentium* are transferred to traditionally susceptible mice, the recipient mice achieve resistance as well (Ghosh et al., 2011; Willing et al., 2011). However, the microbiota is sensitive to disruption by overt inflammatory responses, antibiotic use, and invasion by pathogens. The inflammatory response to infection alters the microbial composition in the gut and may lead to a more permissive environment for the invading pathogen (Lupp et al., 2007). This dysbiosis alters the diversity of the microbiota and reduces the population of commensals while allowing *C. rodentium* to multiple to overwhelming numbers (Lupp et al., 2007).

1.1.2.1 Genetic organization and similarity to EPEC/EHEC

EPEC, EHEC, and *C. rodentium* share 3000 genes out of 5000 encoded by *C. rodentium*, accounting for the similarities in their infection phenotypes and virulence mechanisms, while maintaining approximately 1,500 unique genes (Petty et al., 2010). The *C. rodentium* genome is composed of one bacterial chromosome and four plasmids. The 5.3 Mb genome encodes roughly 5000 predicted coding sequences distributed over 17 genomic islands (Petty et al., 2010). Aside from the LEE encoded T3SS, *C. rodentium* also encodes a type IV pilus and 2 putative T6SSs some of which are shared with either EPEC or EHEC. *C. rodentium* also encodes 29 effector proteins that are delivered into host cells via the T3SS, 22 of which are also found in EPEC and other A/E pathogens (Petty et al., 2010).

Bacteria are capable of acquiring genetic material either from their surrounding environment, phage infection, or through horizontal gene transfer. This leads to the generation of highly variant strains of bacteria that while related induce different pathology via different mechanisms. It was determined that the *C. rodentium* genome shows a high degree of insertional sequences and pseudogenes that suggests that its genome is highly dynamic (Petty et al., 2011). These sequences are also associated with almost half of the shared genes between EHEC and EPEC that are not part of the core *E. coli* genes. The sequence identity and evidence of more recently acquired genes led to the conclusion that EHEC, EPEC, and *C. rodentium* demonstrate convergent evolution. This genetic flux is hypothesized to account for the adaptability of *C. rodentium*, and other A/E pathogens, to their surrounding environment, chiefly the harsh conditions of the gastro-intestinal tract. Petty and colleagues determined that following stressors from the host, prophages within the genome might be activated to induce a different transcriptional profile (Petty et al., 2011). The exact mechanisms, protein mediators, and regulation of these recombination events remain unclear and further work is needed to elucidate these events during infection.

1.1.3 Virulence factors

The LEE pathogenicity island is structured into 5 operons that control 41 genes encoding gene regulators, the genes needed for the structural proteins of the T3SS, the genes needed for bacterial attachment, and the core set of secreted protein effectors (Deng et al., 2001; Goosney et al., 2000). The T3SS encoded by LEE is composed of the structural proteins, EspA, EspB, EspD that function to create the translocon used to translocate bacterial effector proteins from the bacterial cytoplasm into the host cell cytoplasm. Among those effectors, the translocated intimin receptor (tir) is the best studied and induces the formation of pedestals and mediates the strong attachment of these bacteria to host epithelial cells by binding to its receptor intimin on the bacteria (Croxen et al., 2013; Goosney et al., 2000; Mundy et al., 2005). The remaining LEE encoded effectors are primarily involved in assisting in the tight binding of bacteria to the host cell, however some have roles within the host as well. It is through the expression of the LEE virulence genes that *C. rodentium* is able to maintain its close colonization of the epithelial cells during the early stage of infection, bypassing the mucus layer and therefore establishing itself away from the competition of the microbiota (Kamada et al., 2012). However, as the infection peaks around 12-14 days post infection, LEE expression is down-regulated and *C. rodentium* colonization shifts toward to the lumen where they are eventually outcompeted by the microbiota (Kamada et al., 2012).

The regulation of the LEE pathogenicity island is well studied, owing to its importance for A/E lesion formation. The Ler protein promotes the transcriptional activation of the LEE operons, as well as GrlA, another positive regulator, as well as GrlR, the negative regulator of LEE (Deng et al., 2004; Yang et al., 2010). Encoded by the LEE1 operon, Ler expression is controlled by other common transcriptional activators that are stimulated by environmental factors. Ler acts to stimulate the expression of GrlA, which further stimulates the transcription of the remaining LEE encoded genes. GrlR expression is also induced and it negatively regulates LEE by blocking the transcription of the LEE1 operon, limiting the expression of Ler and GrlA (Deng et al., 2004; Yang et al., 2010).

1.1.3.1 T3SS effectors

Aside from the LEE encoded effectors, EPEC, EHEC, and *C. rodentium* encode a variety of non-LEE effectors that have been collected through mobile genetic elements. Of the 29 effectors currently recognized in *C. rodentium*, 22 are shared with EPEC and EHEC (Petty et al., 2010). The flanking DNA sequences surrounding these genes suggest that these were mostly acquired through horizontal gene transfer. The effectors are proteins of diverse functions such as proteases, kinases, adhesions, ligases, etc. (Wong et al., 2011). The functions of these effectors allow for the virulence associated with each of these pathogens, accounting for the shared and unique mechanisms each of these pathogens employ. The fact that *C. rodentium* encodes so many of the same virulence factors as EPEC and EHEC has greatly advanced the study of these pathogens and the understanding of their pathogenesis. Of these 29 effectors, 7 are the LEE effectors related to A/E lesion formation, and the remaining have functions relating to manipulating the host signalling pathways (Petty et al., 2010; Wong et al., 2011). Effectors have been implicated in altering actin polymerization, NF-κB signaling, tight junction maintenance, cell survival and apoptosis, ion transport, and other cell outcomes. Often many effectors will target multiple levels of a particular pathway. For example, NleB, NleC, NleH, and NleE have all been shown to alter various stages of the NF-κB pathway (Baruch et al., 2011; Shames and Finlay, 2012; Wong et al., 2011). Among the variety of effectors, the non-LEE encoded effectors (Nle) NleH and NleC are of particular interest to this thesis due to their interaction with NF-κB signaling and RPS3 (see section 1.2.2.2).

1.1.3.1.1 Kinases NleH1 and NleH2

EPEC and EHEC encode two NleH effectors, NleH1 and NleH2, of which only NleH1 has a homolog in *C. rodentium*. These two effectors share high sequence homology to each other, 84% similar, as well as to the *Shigella* OspG effector, known to alter the NF-κB pathway (Tobe et al., 2006). These proteins have a conserved kinase active site and are PDZ-domain containing proteins (Wong et al., 2011). NleH1 and NleH2 were shown to block apoptosis in infected cells by interacting

with Bax-inhibitor 1 and preventing the action of caspase-3 (Hemrajani et al., 2010). Unlike the OspG effector, which inhibits the NF-κB pathway by preventing the ubiquitination and degradation of IκBα, NleH1 and NleH2 were found to bind to RPS3, a specifier of NF-κB activity (Gao et al., 2009; Wan et al., 2007). It was determined that NleH1 blocks the phosphorylation of RPS3 at serine 209 by IKKβ that is required for its nuclear function in stimulating the expression of a subset of NF-κB genes (Wan et al., 2011). The kinase function of NleH1 is required for its function, however it does not direct phosphorylate RPS3 or IKKβ. Furthermore, NleH1 blocked the activation of an RPS3-dependent reporter plasmid, whereas NleH2 induced the expression of the reporter (Gao et al., 2009). Using NleH1 to selectively manipulate NF-κB gene expression results in an incomplete inflammatory response to infection, and in fact an NleH mutant strain was demonstrated to be hyper-virulent (Gao et al., 2009). As discussed in Chapter 2 and 3 of this thesis, the selective inhibition of the RPS3 branch of NF-κB signaling is a novel mechanism by which A/E pathogens can acutely regulate their microenvironment.

1.1.3.1.2 NleC

NleC is a zinc metalloprotease that has been extensively studied in the last few years. In a series of screening experiments, NleC was identified as down-regulating the expression of IL-8, a key proinflammatory chemokine that recruits neutrophils. As a protease, it was determined that NleC cleaves p65 in order to reduce the activity of the NF-κB pathway within the infected cell (Li et al., 2014; Muhlen et al., 2011; Pearson et al., 2011; Turco and Sousa, 2014; Yen et al., 2010). More detailed mutagenesis experiments determined that the N-terminus of NleC was needed for the interaction with p65 and that NleC contained an HEXXH motif conserved amongst metalloproteases (Muhlen et al., 2011). Chelating zinc ions, such as with EDTA, is sufficient to block the cleavage of p65, as is mutagenesis of the conserved metalloprotease motif (Baruch et al., 2011; Li et al., 2014; Muhlen et al., 2011; Yen et al., 2010). Aside from p65, NleC has also been demonstrated to cleave p300 (Shames et al., 2011), p50 (Muhlen et al., 2011; Pearson et al., 2011; Turco and Sousa,

2014), and IκB (Muhlen et al., 2011). However, the majority of these studies are using over expression systems that utilize ectopically expressed proteins at non-relevant concentrations, which may provide false positive results. Given the sequence homology between p50 and p65 at the Rel homology domain, further examination of the mechanisms by which NleC targets its substrates is required to determine specificity. Moreover, there are discrepancies in the field regarding the cleavage site on p65, with proline 10 and alanine 11 (P10/A11) (Yen et al., 2010), and cysteine 38 and glutamic acid 39 (C38/E39) (Li et al., 2014; Muhlen et al., 2011; Turco and Sousa, 2014) both proposed by different studies. The complete cleavage of p65 within the cell will affect the cell's ability to respond to pathogen infection by blocking activation of inflammatory genes, block anti-apoptotic signaling to protect the cell from death, and alter other cellular pathways. However, NleC only mediates cleavage of a small percentage of p65, leaving a large amount of full-length p65 present within the cell available for activating gene transcription. It is therefore striking that despite only cleaving a small portion of the total p65 present within the cell, there is such a large effect on the transcription of IL-8 as has been measured by multiple studies (Li et al., 2014; Muhlen et al., 2011; Pearson et al., 2011; Turco and Sousa, 2014; Yen et al., 2010). The specific cleavage site and amount of cleavage is of particular interest as the sequence and fate of the cleavage product may have important downstream implications as discussed more thoroughly in Chapters 2 and 3.

1.1.3.2 Phase variation

Aside from directly manipulating host proteins in an effort to subvert host responses to infection, bacteria also use phase variation to alter the expression of antigens on their cellular surface to evade recognition by antibodies (Goldberg et al., 2014). This process of inducible phenotypic heterogeneity involves the reversible rearrangement of genetic elements to turn on and off the expression of select genes (van der Woude and Baumler, 2004). Phase variation is either carried out via the homologous recombination machinery or through recombinases that recognize specific flanking sequences. Studied since the early 1920s, the antigenic variations seen in Salmonella

infections have been attributed to *hin* gene that encodes the Hin-catalyzed site-specific DNA invertase, a serine DNA recombinase that functions as part of an operon (reviewed (Johnson, 2015)). Briefly, the Hin system involves a specific enhancer, two imperfect palindromic sequences, and a promoter. The Hin brings together the enhancer regions along with the two palindromic sequences and mediates the inversion of the intervening DNA sequence. Transcription of the *flagellin* genes depends on the orientation of the promoter sequence, where in the "on" orientation the *FljB* flagellin is expressed along with the repressor for *FljC* flagellin. In the "off" orientation, neither the *FljB* nor the *FljC* repressor is transcribed, allowing for the transcription and translation of the *FljC* gene, respectively (reviewed (Johnson, 2015). This antigenic variation strategy allows immune escape, rendering the antibody response generated toward the original antigen obsolete, giving *Salmonella* a temporary advantage over its host. In *E. coli* the *fim* operon functions a similar way to regulate the expression of fimbriae, a major target for the immune response and a virulence factor that mediates the early stages of bacterial attachment to epithelial cells. It remains to be seen what other virulence factors may be controlled in such a manner.

1.2 Innate responses

As the first line of defense against infection, the innate immune response to the gut microbiota and infection begin to shape the inflammatory response and the outcome of disease. The functions of the innate immune response contribute to the clearance of *C. rodentium* infection and structure the subsequent adaptive immune response (Collins 2014, Liu 2012). Gut epithelial cells, macrophages, dendritic cells, and the innate lymphocytes lining the intestinal mucosa form a barrier that physically separates the microbiota and invading bacteria from the immune system (Peterson 2014). Aside from the physical barrier, these cells also interact with bacteria by producing various anti-microbial peptides, mucin, cytokines and chemokines in response to microbial stimulation.

1.2.1 Maintenance of the mucus barrier

The mucus layer within the colon exists to minimize the direct contact of the colonic epithelial cells with the microbiota to prevent inducing an overwhelming immune response (Peterson and Artis, 2014). In order to maintain this physical barrier, specialized epithelial cells produce and secrete mucin proteins into the luminal space where they form a complex network. There are two layers of mucus covering the entire length of the human colon, which are the loosely attached outer layer and the intimately attached inner layer (Johansson et al., 2011). Studies examining the localization of Muc2, the most abundant mucin protein, demonstrate the difference in density of the outer and inner layers as well as the exclusion of bacteria from the inner layer (Johansson et al., 2011). The outer mucus layer, however, is heavily colonized by the microbiota as it provides both a scaffolding and a nutrient source (Bergstrom et al., 2010; Johansson et al., 2011; Lievin-Le Moal and Servin, 2006; Mizoguchi, 2012). The mucin proteins are heavily glycosylated and form long polymers that along with other proteins, such as trefoil factor 3 (TFF3), secreted into the lumen form a cross-linked and complex network (Johansson et al., 2011). This dense network is relaxed by proteolytic cleavage events as it moves toward to the lumen and more mucin is secreted by goblet cells (Johansson et al., 2011).

Goblet cells produce mucins and anti-microbial peptides in response to IL-22-STAT3 signaling (Lievin-Le Moal and Servin, 2006; Mizoguchi, 2012; Sugimoto et al., 2008). TFF3 and resistin-like molecule-β (RELMβ) are among the anti-microbial peptides that further stimulate epithelial cells and goblet cells in a positive feedback loop, enhancing the barrier integrity by providing repair signals (Peterson and Artis, 2014). However since goblet cells rely on these signals in order to produce mucus, alterations to the microenvironment can have robust effects on the mucus layer. Antibiotic treatment (Wlodarska et al., 2011), infection (Linden et al., 2008), and depletion of IL-22 (Sugimoto et al., 2008), can all affect the production of mucin. Most animal models of spontaneous colitis (such as $Muc2^{-/-}$, $IL10^{-/-}$, or $TLR5^{-/-}$ mice) also have altered mucus

layers (Johansson et al., 2014) emphasizing the importance of this physical and chemical barrier in separating the mucosa from the microbiota. The lack of the mucus layer leads to constant stimulation and ulcerative lesions along the mucosa resulting in barrier permeability and inflammatory responses. The effect of infection and injury on mucus production and the immune mediated regulatory mechanisms inducing mucin production requires more detailed study.

1.2.2 Innate signaling pathways

Various cellular immune receptors, either on the cellular surface or within the cytoplasm, act to initiate signaling cascades that activate diverse aspects of the immune response. These receptors are classified as pattern recognition receptors (PRRs) and they recognize pathogen-associated molecular patterns (PAMPs). PRRs recognize a varied but restricted number of microbial structures that are conserved across many pathogens. Among the PRRs are the Toll-like receptors (TLRs) which sense viral, bacterial, and parasite PAMPs (Janssens and Beyaert, 2003; Mogensen, 2009; Uematsu and Akira, 2006); the Nod-like receptors (NLRs) that sense primarily bacterial PAMPs but also chemical danger signals (Chen et al., 2009; Franchi et al., 2009; Sirard et al., 2007; Wen et al., 2013); and the RIG-I-like receptors (RLRs) that sense nucleic acids, primarily viral RNA (Dixit and Kagan, 2013; Schlee et al., 2009) (reviewed in (Tam and Jacques, 2014)). PRRs stand as the first step in a signaling cascade that involves many scaffold and adaptor proteins that work to transmit and amplify signals from the site of detection (cell surface or endosome) to nucleus to induce a cellular response. Each pathway differs in their use of adaptor molecules and the steps needed to activate a response, yet these pathways all eventually activate a set of transcription factors to cause transcriptional activation of a set of target genes. Effector functions such as inducing the expression of anti-microbial peptides, reactive oxygen species, and chemokine production to recruit inflammatory cells to the site of infection are all induced following activation of innate signaling pathways (Bergstrom et al., 2012; Kawai and Akira, 2010). In the intestinal epithelium, these PRR function to maintain a constant low level of stimulation, from the microbiota, providing important

signals for maintaining homeostasis; in the absence of the microbiota, dysregulation occurs (Rakoff-Nahoum et al., 2004). Homeostatic activation results in the intestinal epithelial and resident immune cells striking a constant balance between basal and inflammatory signalling. In an attempt to strike this balance, PRRs responsiveness is segregated along the polarized epithelial cell layer, with apical (luminal) stimulation resulting in very little activation or tolergenic responses, whereas basolateral stimulation results in the inflammatory activation (Blander and Sander, 2012; Gewirtz et al., 2001; Lee et al., 2006; Rhee et al., 2005).

1.2.2.1 TLRs, NODs, and sensing bacterial infection

The TLRs are transmembrane protein receptors that recognize pathogens at either the cellular surface (TLR1, TLR2, TLR4, TLR5, TLR6) or from within endosomes (TLR3, TLR7, TLR8, TLR9) (Barton and Kagan, 2009). TLR1, TLR2, TLR4, TLR5, and TLR6 detect bacterial products such as lipoproteins and flagellin (Tam and Jacques, 2014). The other TLRs primarily sense nucleic acids that originate from bacteria, viruses, or parasites (Albiger et al., 2007; Bafica et al., 2006; Barber, 2011; Barton, 2007; Tam and Jacques, 2014). Following extracellular (or endosomal) receptor engagement, the TLRs dimerize leading to the auto-phosphorylation of their cytosolic Toll/Interleukin 1 receptor (TIR) domains. The following events are dependent on the specific TLR involved and the stimuli. The TLR's TIR domains will interact with the TIR domains of either of two adaptor molecules, myeloid differentiation primary response gene 88 (MyD88) or TIR-domain-containing adapter-inducing interferon-β (TRIF). For the purposes of this thesis we will focus on TLR2 and TLR4, whose involvement has been reported in response to *C. rodentium* infection (Gibson et al., 2008). TLR2 forms heterodimers with TLR1 and TLR6 giving it a broader range of bacterial lipid detection and ultimately activates NF-κB. In contrast, TLR4 forms homodimers and specifically detects Gram-negative bacterial lipopolysaccharide (LPS) leading to the downstream activation of NF-κB and IRFs (Kagan et al., 2008). Both TLR2 and TLR4 signal through the MyD88 adaptor protein (Kawai et al., 1999; Takeuchi et al., 2000), which interacts with several interleukin-1 receptor-

associated kinase (IRAKs) proteins, then TNF-receptor-associated factor 6 (TRAF6) and a series of other kinases and ubiquitin conjugating enzymes that result in the activation of the MAPK (leading to p38 and JNK activation) and NF-κB pathways (Chen, 2005). TLR4 can also signal independent of MyD88, using the TRIF adaptor, leading to the downstream activation of IRF3 and IRF7 (Hirotani et al., 2005; Yamamoto et al., 2003). The activation of these transcription factors leads to inflammatory immune responses engineered to aid in clearing infections, albeit these immune responses can also induce some pathology (Bhullar et al., 2015).

The role of TLR4 in activating immune responses during systemic infection has demonstrated its importance in limiting disease spread (Campos et al., 2004; Mann et al., 2004; Miyake, 2004; O'Brien et al., 1982; Schilling et al., 2003; Vazquez-Torres et al., 2004). However, during *C. rodentium* infection in the gut, TLR4 was revealed to have an immunopathogenic effect. Immunopathology is being recognized more and more for causing a lot of the damage to the host previously associated with infections (Karupiah and Chaudhri, 2007; Yang et al., 2013). Namely, TLR4 expressing mice experienced more severe disease as measured by tissue pathology and infiltrating immune cells. Moreover, the spread of *C. rodentium* through the colon was slowed in TLR4$^{-/-}$ mice, taking longer to reach high numbers in the distal colon (Khan et al., 2006). Despite the delay in colonization, TLR4$^{-/-}$ mice were still able to clear the infection at a similar rate as wild-type mice, likely due to other compensatory signaling pathways. TLR4 signaling was found to be important for the transcriptional activation of two chemokines, MIP-2 and MCP-1. This results in the recruitment of macrophages and neutrophils in response to infection (Khan et al., 2006). This unexpected effect of TLR4 deficiency may have more to do with the low level of TLR4 expression in colonic epithelial cells, which are the primary site of *C. rodentium* infection. In contrast, the high levels of TLR4 expression in macrophages may yield the greater effects of TLR4 in systemic disease (Schilling et al., 2003; Vazquez-Torres et al., 2004). Similarly, in TLR4-/- mice, less severe inflammation occurs in the dextran sodium sulfate (DSS) model of intestinal inflammation. This effect was attributed to decreased chemokine expression, despite an overall increase in morbidity in

DSS-treated TLR4$^{-/-}$ mice (Fukata et al., 2005; Rakoff-Nahoum et al., 2004). The differences in responses in these inflammatory models may stem from the mechanisms by which these models induce tissue injury.

Conversely, the role of TLR2 in regulating *C. rodentium* infection in the mouse intestinal tract was explored utilizing the TLR2$^{-/-}$ mouse TLR2 was hypothesized to be necessary for inducing the activation of the immune response from the lipoproteins produced by the bacteria (Gibson et al., 2008). Interestingly, during infection TLR2$^{-/-}$ mice induced a similar profile of inflammatory cytokines and chemokines to wild-type mice with *C. rodentium* infection (Higgins et al., 1999; Simmons et al., 2002), indicating that TLR2 signaling does not play a role in clearing the pathogen (Gibson et al., 2008). However, TLR2$^{-/-}$ mice were deficient in their expression of IL-6 post-infection and maintaining the proper localization of tight junction proteins, both during infection and under basal conditions, which is known to be affected during *C. rodentium* infection (Cario et al., 2004, 2007; Conlin et al., 2009; Gibson et al., 2008). The devastating effect of TLR2 deficiency after C. rodentium infection uncovered the importance of maintaining the integrity of the epithelial barrier and the role of IL-6 for protecting against widespread epithelial apoptosis. These aspects of gut mucosal immunity were later confirmed by other studies (Conlin et al., 2009; Dann et al., 2008; Guttman and Finlay, 2009). Therefore, it was determined that TLR2 plays a vital support role in minimizing the damage to the *C. rodentium* infected epithelial cell layer by preventing apoptosis and supporting barrier integrity.

The conflicting effects of TLR4 and TLR2 in response to *C. rodentium* infection are indicative of how important it is to maintain a balanced response to infection. Robust activation of TLR4 leads to the production chemokines that recruit immune cells to the site of infection, however, this costs the host by also inducing tissue damage. The activation of TLR2 protects the host from overwhelming damage by inducing the expression of IL-6 that exacts anti-apoptotic effects on the epithelial cells. In this way the inflammatory TLR4 response and the protective TLR2 response

balance each other. Aside from invading pathogens, the colonic epithelial cells and resident macrophages are constantly exposed to and stimulated by the microbiota and these low level, balanced interactions are critical for maintaining homeostasis in the gut. The microbiota stimulate the proliferation of intestinal epithelial cells as well as their production of anti-microbial peptides (Khoury et al., 1969; Nenci et al., 2007; Rakoff-Nahoum et al., 2004; Reikvam et al., 2011; Wu and Wu, 2012).

Unlike the TLRs which exhibit more restricted expression and activity within the gut epithelia, the nucleotide-binding oligomerization domain (NOD) and leucine-rich repeat-containing proteins (NLRs) are thought to play the primary role of immune activation in the gut. The NLRs are cytoplasmic sensors of infection, detecting nucleic acids from invading pathogens from all classes as well as other PAMPs. While there are many NLRs and not all are fully characterized, in terms of bacterial immune responses, Nod1 and Nod2 recognize peptidoglycan molecules present within the host cytoplasm (Kanneganti et al., 2007). Nod1 primarily recognizes Gram-negative bacteria (and a few Gram-positive species), whereas Nod2 recognizes both Gram-negative and Gram-positive bacteria (Girardin et al., 2003a; Girardin et al., 2003b; Girardin et al., 2003c). Nod1, thought to act as sensor of the microbiota present within the gut and expressed in both hematopoietic cells and intestinal epithelial cells (Kim et al., 2004), recognizes the release of peptidoglycan that occurs during rapid bacterial proliferation (Goodell and Schwarz, 1985; Rosenthal et al., 1987; Vance et al., 2009). Peptidoglycan is taken up by cells via endocytosis (Lee et al., 2009), phagocytosis of the infecting bacteria (Greenberg and Grinstein, 2002; Stuart and Ezekowitz, 2005; Underhill and Ozinsky, 2002), host peptide transporters (Daniel and Kottra, 2004; Lee et al., 2009; Vavricka et al., 2004). This is further supported by a study demonstrating that Nod1$^{-/-}$ mice have an exponential expansion of their gut microbiota numbers (Bouskra et al., 2008).

Much like the TLRs, the specific arrangement of adaptor proteins and the specific stimulating signal will determine the response induced by activating the Nod1 and Nod2 pathways.

Both Nod1 and Nod2 interact with the RIP2 kinase, via their caspase activation and recruitment domains (CARD), leading to the downstream activation of NF-κB and MAPK and the transcriptional activation of inflammatory cytokines (Hasegawa et al., 2006; Kobayashi et al., 2002; Park et al., 2007a). The inflammasome, composed of NLRC4/ASC/caspase-1, is activated via flagellin molecules delivered into the cell via the T3SS. Upon activation, it cleaves IL-1β and IL-18 from the pro-form to the active form (Franchi et al., 2006; Hersh et al., 1999; Miao et al., 2006; Miao et al., 2008). The various combinations of NOD domain and CARD domain containing proteins that form the NLR signaling pathway provide specificity in the response induced by activation of these proteins.

The role of NLRs has been studied in the context of *C. rodentium* infection as a means to understand the inflammatory responses that could also lead to inflammatory bowel disease (IBD) since nucleotide polymorphisms have been associated with increased risk of IBD (Cho, 2008). Studies utilizing Nod1$^{-/-}$ and Nod2$^{-/-}$ double knockout mice demonstrated deficient early Th17 responses, leading to the conclusions that the NLRs are important for the induction of the innate Th17 response following infection (Geddes et al., 2011). Moreover, they observed less inflammation and pathology at the early time points following infection in the Nod1$^{-/-}$ and Nod2$^{-/-}$ mice which was followed by a enhanced inflammatory response at the later time points. This suggests that early iTh17 cells are important for controlling the infection at the later stages (Geddes et al., 2011). Similarly, in Nod2$^{-/-}$ mice, there is an increase in the bacterial load at early time points resulting in prolonged infection and a high degree of inflammation at later time points during the course of the infection, compared to wild-type mice (Kim et al., 2011). These effects were attributed to deficient trafficking of inflammatory monocytes into the colon that the authors speculated limited direct killing of the bacteria and impaired a monocyte-directed Th1 response needed to drive antibody production and clearance (Bry and Brenner, 2004; Bry et al., 2006; Kim et al., 2011).

1.2.2.2 The NF-κB pathway

The NF-κB signaling pathway is a major regulator of the cellular response to stimuli of all kinds (Hayden and Ghosh, 2012; Li and Verma, 2002; Rahman and McFadden, 2011; Sun and Andersson, 2002; Vallabhapurapu and Karin, 2009; Wan and Lenardo, 2009, 2010). Many of the previously discussed extracellular receptors initiate signaling cascades that converge on NF-κB signalling for inducing the activation of many genes. There are two main activation pathways that result in the activation of NF-κB- responsive genes; the classical pathway and the alternative pathway.

The classical pathway begins with the activation of the trimeric IκB kinase (IKK) complex, containing the IKKα, IKKβ, and IKKγ (also called NEMO; NF-κB essential modulator) subunits. The activated IKK complex in turn phosphorylates IκBα (inhibitor of NF-κB) and induces its degradation by the ubiquitin-proteasome pathway (Chen, 2005). This releases the NF-κB dimer, uncovering its nuclear localization signals (NLS) allowing for its translocation into the nucleus. The NF-κB dimer is typically a hetero- or homodimer composed of two members of the Rel domain family subunits: p65 (RelA), RelB, c-Rel, p50, and p52. The Rel-homology domain mediates protein-protein interactions and DNA binding. Of these, only p65, c-Rel, and RelB contain transcription activation domains (TAD) that initiate transcription (Hayden and Ghosh, 2012). More recently, a non-Rel containing subunit was discovered to form part of some NF-κB complexes. Ribosomal protein S3 (RPS3) is found associated with the NF-κB complex inhibited by IκBα (Wan et al., 2007; Wan et al., 2011). When the NF-κB complex is released for nuclear translocation, RPS3 is also phosphorylated and translocates into the nucleus where it enhances the binding affinity of p65/p50 heterodimers at specific NF-κB sites (Wan et al., 2007). Since then, other co-activating proteins have been found to interact with the NF-κB subunits to mediate the expression of a particular sub-set of genes over others (Fu et al., 2013; Zhang et al., 2014). These studies have begun to shed light on the elusive regulatory mechanisms regarding promoter selectivity and specificity that have lingered in the

NF-κB field for many years. Many of the classical pathway's target genes are involved in inflammatory signaling, immune activation, proliferation, and apoptosis. This makes the classical pathway a prime target of many invading pathogens in order to evade the immune response (discussed in thorough detail in Chapters 2 and 3).

The alternative (non-canonical) activation pathway of NF-κB involves the stabilization and activation of the NF-κB-inducible kinase (NIK) (reviewed in (Sun, 2011)). When NIK is stabilized (Liao et al., 2004), it activates IKKα and induces the processing of p100 into p52 (Senftleben et al., 2001; Xiao et al., 2001; Yilmaz et al., 2003) allowing for the RelB/p52 heterodimer to enter the nucleus (Rahman and McFadden, 2011; Vallabhapurapu and Karin, 2009) and activate target gene transcription.

1.2.2.2.1 Promoter Specificity

The question of how NF-κB complexes generate specific and discrete responses following different stimuli has loomed in the field, until recently. Interactions with other transcription factors and post-translational modifications have demonstrated effects on the transcriptional activation of NF-κB (reviewed in (Oeckinghaus et al., 2011)). In 2007, it was discovered that a non-Rel protein, RPS3, selectively regulates the transcriptional activation of a subset of NF-κB genes. RPS3 was termed a "specifier" and since then more work has been done to uncover the exact function and mechanism of RPS3 dependent selectivity, as well as other "specifier" proteins.

RPS3 is a KH domain containing protein that can bind RNA and DNA (Siomi et al., 1993). In drosophila, RPS3 was shown to be able to cleave DNA at damaged residues and it was therefore thought to have a role in DNA repair (Wilson et al., 1993). Indeed, this was confirmed when RPS3 was shown to remove 8-oxo-G lesion following oxidative stress (Deutsch et al., 1997; Yacoub et al., 1996). Aside from its function in the ribosome, RPS3 was found within the p65/p50 cytoplasmic complex as well as within the nucleus. Containing a nuclear localization sequence, RPS3 was

demonstrated to shuttle back and forth following phosphorylation similar to p65 and to interact with importin α (Wan et al., 2011). Phosphorylation-induced nuclear translocation abilities were also demonstrated for RPS3 following DNA damage (Yadavilli et al., 2007). The interaction of RPS3 and the p65/p50 heterodimer in the nucleus was found to stabilize p65/p50 binding at the promoters of specific NF-κB genes in T cells (Wan et al., 2007). This was confirmed when knock down of RPS3 expression resulted in the decreased activation of select genes, while not affecting the nuclear translocation of p65 or the transcription of other NF-κB genes (Wan et al., 2007). Importantly, knock down of RPS3 by siRNA targets the ribosome free pool of RPS3, presumably the population of RPS3 molecules capable of interacting with NF-κB, without affecting the ribosomal pool of RPS3. Subsequent work demonstrated that aside from certain inflammatory genes such as IL-2 and IL8 (Wan et al., 2007), the transcription of a subset of anti-apoptotic genes are also RPS3 dependent and prevented islet cell death (Mokhtari et al., 2009), such as Bcl-Xl, cIAP2, TRAF, and A20 (Mokhtari et al., 2009; Sen et al., 2012). This study also determined that the p65- cysteine 38 residue has a sulfhydration modification that strengthens the interaction with RPS3 (Sen et al., 2012).

A/E pathogen infection has been shown to abrogate the interaction between p65 and RPS3 via the NleH1 and NleH2 effector proteins discussed previously. While both effectors can bind to RPS3, only NleH1 was shown to inhibit the nuclear translocation of RPS3 (Gao et al., 2009). This inhibition occurs by blocking the phosphorylation of RPS3 by IKKβ (Gao et al., 2009; Wan et al., 2011). This resulted in the selective inhibition of inflammatory responses, but not global suppression of NF-κB genes. Similarly, previous work from our lab has shown that overexpression of an N-terminal fragment of p65 (containing the C38 residue) is capable of successfully sequestering RPS3 away from full length p65 and therefore block downstream p65-RPS3 dependent gene transcription (Wier et al., 2012). Furthermore, during apoptosis caspase-3 cleaves p65 generating an N-terminal fragment containing amino acids 1-97. This fragment is also capable of blocking the RPS3-p65 interaction by preventing the nuclear translocation of RPS3. In this model, an N-terminal fragment

of p65 containing the C38 residue needed for binding RPS3 is sufficient to sequester RPS3 in the cytoplasm preventing its nuclear function (Wier et al., 2012). While more work is needed to fully outline all of the p65-RPS3 dependent genes, it is clear that this axis of NF-κB signaling plays a very important role in multiple infection settings. This topic is revisited in chapter 2.

Sam68 is another KH domain containing protein that has a wide range of functions from splicing, RNA stabilization, adipogenesis, regulating fertility, and carcinogenesis (Bielli et al., 2011; Huot et al., 2012; Iijima et al., 2011; Lukong and Richard, 2003; Paronetto et al., 2009). Sam68 is a signal transduction activator of RNA (STAR) member and as an RNA binding protein its function modulates many cellular pathways (Lukong and Richard, 2003; Sette et al., 2010). Previous work by our group has defined a role for Sam68 in regulating the transcription of a subset of NF-κB genes in T cells, different from those regulated by RPS3 (Fu et al., 2013). The mechanism of Sam68 regulating NF-κB gene transcription differs from RPS3, because Sam68 is a predominantly nuclear protein. It was therefore demonstrated that Sam68 is activated in the nucleus by phosphorylation by IKKα, which translocates to the nucleus following T cell receptor engagement. Following phosphorylation Sam68 was found to mediate and strengthen p65/p50 DNA binding to the promoter for CD25, the α chain of the IL-2R (Fu et al., 2013). Like in the RPS3 studies, this was confirmed by showing a lack of CD25 transcriptional activation following siRNA knock down of Sam68.

Kruppel-like factors (KLFs) are another branch of transcription factors that have roles in stimulating responses involved in cell development and homeostasis. Among these, KLF-6 was shown to regulate the transcriptional activity of NF-κB following TNF-α and IL-1β stimulation (Zhang et al., 2014). They showed that KLF-6 interacts with p65 within the nucleus and is present at the promoters of NF-κB downstream genes with p65. Based on the known consensus site for each of these proteins, it is likely that p65 and KLF-6 are targeting the same DNA sequences, as their

consensus sequences are reverse complements of each other (Zhang et al., 2014). This represents another novel mechanism by which NF-κB promoter selectivity is achieved.

1.2.3 Innate immune cells and infection

Resident innate immune cells in the gut typically maintain a regulatory, anti-inflammatory phenotype to prevent the over stimulation of the tissue to the normal microbiota. Macrophages present in the lamina propria, just under the epithelial cell layer in the gut, constitutively express IL-10 (Denning et al., 2007). Moreover, in response to pathogen infection these cells induce less of a proinflammatory Th1 response than their bone marrow counterparts (Monteleone et al., 2008). Similarly, gut resident dendritic cells induce more of a Th17 like response following stimulation rather than a Th1 response (Monteleone et al., 2008; Schulz et al., 2009). The microbiota have been shown to be critical for the development of appropriate responses to infection. Following *C. rodentium* infection however, the stimulation of TLRs and NLRs as discussed above, induce the production of inflammatory mediators such as IL-6, IL-12, TNF, and IL-23 as well as IL-1β and IL-18 (Clarke and Weiser, 2011). Stromal cells within the gut, also expressing PRRs are induced to produce chemokines, such as CCL2 and CXCL1, that result in the recruitment of neutrophils to the site of infection to directly kill and control the spread of infection (Borregaard, 2010; Kim et al., 2011; Park et al., 2007b; Spehlmann et al., 2009). These inflammatory innate cells induce the robust T cell responses that are both Th1 and Th17 in nature.

Innate lymphoid cells (ILCs) compose a more recently described cell type that has shown to be critical in the maintenance of mucosal homeostasis and found to be present at multiple barrier sites (Kim et al., 2013; Monticelli et al., 2012; Tait Wojno and Artis, 2012). These cells mount responses to pathogens and injury while lacking the antigen-specific receptors of traditional CD4+ T helper (Th) cells (Spits and Cupedo, 2012). Similar to the classical Th cells, ILCs have varying functional phenotypes induced by the cytokine milieu that have been categorized as group 1 (akin to

the Th1 proinflammatory cells), group2 (Th2-like in response and development), and group 3 (Th17-like with proinflammatory and protective properties) (Peterson and Artis, 2014; Spits et al., 2013; Tait Wojno and Artis, 2012).

Within the gut, the group 3 ILCs have been demonstrated to play an important role in maintaining barrier integrity and control of the microbiota, while group 2 ILCs are responsible for important roles during helminth infection, unsurprisingly due to their Th2-like phenotype (Peterson and Artis, 2014; Tait Wojno and Artis, 2012). Given the documented role of group 3 ILCs, particularly the RORγt^{+} ILCs, in intestinal barrier maintenance and inflammatory responses within the gut, their role in response to A/E pathogen infection has begun to be characterized. These cells are a constant source of IL-22, in response to IL-23, within the gut and in that way reinforce the barrier by stimulating the epithelial cell expression of anti-microbial peptides and goblet cell mucus production (Sanos et al., 2009; Satoh-Takayama et al., 2009; Sonnenberg et al., 2011). DSS-induced injury and inflammation induces the expansion of RORγt^{+} ILCs and their production of IL-22 in an attempt to limit local inflammation and prevent commensal systemic dissemination (Sawa et al., 2011; Sonnenberg et al., 2012). Likewise, several studies have indicated that during *C. rodentium* infection group 3 ILCs, in an IL-23 dependent manner, are major producers of IL-22 independent of a functional lymphocyte compartment (Cella et al., 2009; Satoh-Takayama et al., 2009; Sonnenberg et al., 2011; Zheng et al., 2008). The rapid lethality experienced by IL-22$^{-/-}$ mice underscore the importance of this cytokine, and therefore these cells, for protecting against intestinal inflammation. However, the dual nature of IL-22 has also led to studies examining the capacity of group 3 ILCs to exacerbate IBD in both mouse models and human patients. The pathogenic capacity of ILCs appears to be context dependent in murine models (Ahern et al., 2008; Monteleone et al., 2012), though further work is needed to confirm these phenotypes.

Apart from IL-22, cells resembling ILCs and producing IL-17 have been isolated from human patient samples (Geremia et al., 2011). Moreover, the discovery of an innate Th17 like cell type that was responding to *C. rodentium* infection via NLR receptors and was critical for driving the neutrophil response demonstrates that ILCs are variable in their phenotypes (Geddes et al., 2011). The IL-17-family of cytokines is composed of six members (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F), which are produced following the stimulation of Th17 responses. Of those, the functions of IL-17A and IL-17F have been most thoroughly studied. This cytokine family has proinflammatory functions inducing the expression of chemokines by epithelial cells resulting in the downstream recruitment of neutrophils (Geddes et al., 2011; Jin and Dong, 2013). Produced mainly by Th17 T cells and ILCs such as the iTh17, IL-17 is typically produced at early and late stages of infection and is critical for the clearance of *C. rodentium* from the host (Kim et al., 2011). The early production of IL-17 and its downstream function serve to control the growing population of invading *C. rodentium* (Geddes et al., 2011; Kim et al., 2011).

1.2.4 IL-22

IL-22 is a member of the IL-10 family of cytokines that has demonstrated protective, reparative effects on tissue as well as pathogenic and inflammatory effects, depending on the context. IL-22 also serves as a bridge between the immune response and the epithelial cells, as it is expressed by T cells and innate lymphocytes (Ouyang et al., 2011; Rutz et al., 2013), yet mainly epithelial cells express the cognate receptor (Wolk et al., 2004). The dimeric receptor is composed of the IL-10R2 β chain (shared by many members of the IL-10 family) and the IL-22R1 as the α chain (Kotenko et al., 2001). T cells and ILCs produce IL-22 following IL-6 and IL-23 stimulation (Trifari et al., 2009; Zheng et al., 2008). In the colon, ILCs are the major source of IL-22, particularly under infection conditions (Cella et al., 2009; Satoh-Takayama et al., 2009; Sonnenberg et al., 2011).

IL-22 acts to reinforce epithelial cells by stimulating the production of mucin proteins and other host defense mechanisms (Sugimoto et al., 2008; Zenewicz et al., 2013). The mucin layer in the gut physically separates the microbiota from the epithelial cells to prevent the over stimulation and activation of the epithelial innate responses within the gut. Consequently, Il-22$^{-/-}$ mice have an altered microbiota that is transferable to co-housed wild-type mice, leaving them more susceptible to colitis models (Zenewicz et al., 2013). IL-22 also stimulates the production of anti-microbial peptides such as alarmins, defensins, and the Reg proteins to directly kill or prevent the further growth of pathogenic bacteria (Liang et al., 2006; Zheng et al., 2008). These antimicrobial peptides target broadly conserved bacterial structures causing pore formation in the bacterial cell walls, killing the bacteria (Peterson and Artis, 2014).

In this way, IL-22 induces protective responses following injury or inflammation, and given the lethality following *C. rodentium* infection in IL-22$^{-/-}$ (Sugimoto et al., 2008; Zheng et al., 2008), the protective stimuli IL-22 provides are of great importance. IL-22$^{-/-}$ mice experience profound damage to the intestinal epithelial following infection, resulting in the systemic spread of *C. rodentium* to other organs (Zheng et al., 2008). The mechanism behind this lethality and the mechanisms by which pathogens may regulate the expression of IL-22 during infection remain to be elucidated.

Despite the protective roles of IL-22, high levels of IL-22 have also been linked to Crohn's disease and ulcerative colitis (Andoh et al., 2005; Brand et al., 2007; Wolk et al., 2007). Since, IL-22 also induces the production of proinflammatory cytokines it has been hypothesized that this response can lead to immunopathology. Moreover, a pathogenic role of IL-22 has been best described in inflammatory skin models of disease such as psoriasis, and linked to the increased expression of other inflammatory cytokines (Lowes et al., 2007). The stimulatory and proinflammatory effects of IL-22 have also linked the expression or dysregulation of this cytokine with cancer, with increasing reports associating higher expression of IL-22 in various cancer settings (Jiang et al., 2013; Kim et al., 2014; Petanidis et al., 2013; Peterson and Artis, 2014; Xu et al., 2014).

Indeed, IL-22 overexpression in gastric cancer promoted proliferation signals and the migration of cancer cells (Ji et al., 2014). Thus, like most immune compartments, the careful regulation of this immune axis is imperative to prevent inducing immunopathology or oncogenic outcomes.

1.3 Adaptive immune responses to *C. rodentium*

The adaptive immune response is greatly shaped by the preceding innate immune response and is subject to the signals already established during the early part of the infection. The great power of the adaptive immune response is the ability to generate highly specific responses to invading pathogens through the use of genetic recombination events for the genes encoding their receptors. These responses allow for more than the detection of self vs. non-self, but in fact allow for the generation of memory to specific pathogens, preventing re-infection. These responses are also amplified by the innate immune response and are crucial for providing sterilizing immunity to many invading pathogens. In terms of *C. rodentium*, CD4 T cells and B cells are critical to control and clear the infection from the gut. Studies using RAG1$^{-/-}$ mice, which have no B and T cells, maintained high bacterial burden for weeks post infection and were ultimately unable to control or clear the infection (Simmons et al., 2003; Vallance et al., 2002). While these studies did not observe mortality following infection, the extensive pathology and huge bacterial burden demonstrate the importance of the adaptive immune response following infection.

1.3.1 T cells

It is well established that the type of CD4 T cell response to infection will shape the outcome of the infection and the B cell response. As naïve T cells are stimulated and activated the cytokine milieu plays an important role in guiding the phenotypic profile the CD4 T cell response will take. Typically, Th1 cells develop following exposure to IFNγ through the stimulation of T-bet and STAT1 to further drive the production of IL-12 and perpetuate the Th1 response (Mullen et al., 2001). Th2 cells on the other hand develop in IL-4 rich environments activating the transcription

factors STAT6 and GATA-3, inducing the expression of more IL-4 (Zheng and Flavell, 1997). Th17 cells develop in the context of IL-6 and TGF-β secretion with the involvement of STAT3 and RORγt transcription factors (Ivanov et al., 2006; Jin and Dong, 2013). Each of these profiles has been characterized to guide the immune response to specific inflammatory insults such as the inflammatory Th1 response to bacteria, parasitic infection, and viruses, or the anti-inflammatory Th2 response needed for helminth infection clearance.

The T cell response to *C. rodentium* infection is vital to the control and clearance of the pathogen as mice deficient in CD4 T cells, are extremely susceptible to infection and experience high mortality (Simmons et al., 2003; Vallance et al., 2002). While these mice experienced less inflammation at the site of infection, they were unable to clear the infection and maintain their barrier integrity resulting in the systemic spread of infection. These studies demonstrated that the CD4 T cell response to infection aided the production of a humoral response that is critical for the control and clearance of *C. rodentium* (Simmons et al., 2003). Initially following *C. rodentium* infection, there is a robust Th1 response characterized by the production of IL-12 and IFNγ, that later is surpassed by a Th17 response following the production of IL-23 from antigen-presenting cells (Higgins et al., 1999; Khan et al., 2006; Shiomi et al., 2010). The Th1 response has been demonstrated to stimulate macrophage phagocytosis at the site of infection as well as promote the B cell antibody response in an IFNγ-dependent manner (Shiomi et al., 2010).

Importantly, the *C. rodentium* infection model has been used to dissect out the function and significance of the Th17 and Th22 immune responses. The Th17-mediated IL-17 secretion at early and late stages of the *C. rodentium* response are critical for controlling the bacterial burden (Ishigame et al., 2009; Jin and Dong, 2013). Th17 cells are CD4 T helper cells that differentiate following TGF-β and IL-6 signaling and secrete IL-17 and IL-22 and can exert both protective and pathogenic

effects depending on the context of stimulation. The combinatory effect of IL-17 recruiting neutrophils for clearing bacteria and the IL-22 effect on epithelial cell to induce the production of anti-microbial peptides demonstrate how these responses work in synergy to achieve clearance. In 2012, it was determined that Th22 cells that developed in presence of IL-6 but not TGFβ are important and potent producers of IL-22, but not IL-17, in the context of infection with *C. rodentium* at later stages of infection (Basu et al., 2012), taking over for role of the iTh17 and ILCs (Geddes et al., 2011; Sawa et al., 2011; Sonnenberg et al., 2012).

1.3.2 B cell

The B cell branch of the adaptive immune response functions to limit the spread of the invading pathogen by secreting antibodies that physically attach to protein epitopes on the pathogen that match the antibody variable regions. Along the gut, IgA secreting plasma cells are constantly releasing IgA into the lumen to keep control over the microbiota (Peterson and Artis, 2014; Wu and Wu, 2012). In the context of *C. rodentium* infection, it is well known that IgG and IgA responses are induced against bacterial proteins (Frankel et al., 1996; Ghaem-Maghami et al., 2001). Studies that examined the role of CD4 T cells in response to *C. rodentium* infection also observed that when CD4 T cells were depleted, reduced antibody responses also occurred (Simmons et al., 2003). These studies were followed up using B cell deficient mice (μMT mice that lack IgG and IgM responses), which demonstrated that similar to CD4 T cell deficiency, these mice were unable to control infection with C. *rodentium* (Simmons et al., 2003). However, the systemic transfer of immune serum was able to restore control of infection, despite not altering the mucosal response. Subsequently, it was determined that secretory antibodies, IgA and IgM, were dispensable for controlling the infection (Maaser et al., 2004). This is particularly intriguing since the mucosal response to infection involves the secretion of a great deal of IgA and the bacteria are residing right at the epithelial-luminal surface (Frankel et al., 1996). More recently, it was determined that mice generated antibodies

that specifically recognized LEE-encoded virulence factors which labeled virulent bacteria in the lumen of infected mice (Kamada et al., 2015). During the highly colonized, early infection phase there is a high expression of the LEE encoded virulence genes. This expression is down-regulated as the infection peaks and begins to clear, leading to the rise of avirulent *C. rodentium* in the lumen (Kamada et al., 2012). While it remains unclear what leads to the down-regulation of LEE, it is hypothesized that these antibody-bound bacteria are then selectively engulfed by neutrophils, as they are required for mediating clearance of infection (Kamada et al., 2015).

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2 Metalloprotease NleC suppresses host NF-κB/inflammatory responses by cleaving p65 and interfering with the p65/RPS3 interaction

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2.1 Abstract

Attaching/Effacing (A/E) pathogens including enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC) and the rodent equivalent *Citrobacter rodentium* are important causative agents of foodborne diseases. Upon infection, a myriad of virulence proteins (effectors) encoded by A/E pathogens are injected through their conserved type III secretion systems (T3SS) into host cells where they interfere with cell signaling cascades, in particular the nuclear factor kappaB (NF-κB) signaling pathway that orchestrates both innate and adaptive immune responses for host defense. Among the T3SS-secreted non-LEE-encoded (Nle) effectors, NleC, a metalloprotease, has been recently elucidated to modulate host NF-κB signaling by cleaving NF-κB Rel subunits. However, it remains elusive how NleC recognizes NF-κB Rel subunits and how the NleC-mediated cleavage impacts on host immune responses in infected cells and animals. In this study, we show that NleC specifically targets p65/RelA through an interaction with a unique N-terminal sequence in p65. NleC cleaves p65 in intestinal epithelial cells, albeit a small percentage of the molecule, to generate the $p65^{1-38}$ fragment during *C. rodentium* infection in cultured cells. Moreover, the NleC-mediated p65 cleavage substantially affects the expression of a subset of NF-κB target genes encoding proinflammatory cytokines/chemokines, immune cell infiltration in the colon, and tissue injury in *C. rodentium*-infected mice. Mechanistically, the NleC cleavage-generated $p65^{1-38}$ fragment interferes with the interaction between p65 and ribosomal protein S3 (RPS3), a 'specifier' subunit of NF-κB that confers a subset of proinflammatory gene transcription, which amplifies the effect of cleaving only a small percentage of p65 to modulate NF-κB-mediated gene expression. Thus, our results reveal a novel mechanism for A/E pathogens to specifically block NF-κB signaling and inflammatory responses by cleaving a small percentage of p65 and targeting the p65/RPS3 interaction in host cells, thus providing novel insights into the pathogenic mechanisms of foodborne diseases.

2.2 Author Summary

The nuclear factor kappaB (NF-κB) signaling pathway is crucial for host defense, as it orchestrates both innate and adaptive immune responses. Beyond the best-studied Rel proteins (p65, RelB, c-Rel, p50 and p52), RPS3 has been recently identified as a "specifier" component of NF-κB, modulating the promoter selectivity and transcriptional specificity of NF-κB. In particular, the RPS3/p65-conferred signaling pathway was recently shown to play a critical role in host proinflammatory transcription and immune responses. Attaching and effacing (A/E) pathogens and others have acquired sophisticated mechanisms to modulate host NF-κB signaling pathways. We have found that NleC, a metalloprotease effector secreted by A/E pathogens, modulates host NF-κB signaling and inflammatory responses through a novel mechanism. NleC specifically recognizes and cleaves a small percentage of p65 and the generated N-terminal fragment of p65 interferes with the p65/RPS3 interaction, thereby amplifying the effect of cleaving only a small percentage of p65 molecules to selectively inhibit NF-κB gene expression. Our findings highlight a previously unappreciated mechanism through which pathogen-encoded proteases interfere with signaling cascades and inflammatory responses in host cells.

2.3 Introduction

Foodborne diseases caused by enteric pathogens remain a significant and common health threat and an immense economic burden worldwide (Clarke, 2001). Among the causative agents of foodborne illness, diarrheagenic strains of *Escherichia coli* including enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC), typically cause diarrhea, hemorrhagic colitis, and pediatric renal failure (Kaper et al., 2004). EPEC, EHEC, and the rodent-specific pathogen *Citrobacter rodentium* produce characteristic attaching/effacing (A/E) lesions on the host intestinal epithelium after they adhere to these cells (Donnenberg et al., 1997). These pathogens translocate a variety of virulence

proteins (effectors), through a conserved type III secretion system (T3SS), into intestinal epithelial cells (IECs) to modulate host cell functions to the pathogen's advantage (Coburn et al., 2007; Hueck, 1998). An ever-expanding repertoire of T3SS secreted effectors, termed non-LEE-encoded (Nle) effectors, was recently identified in A/E pathogens (Deng et al., 2010; Deng et al., 2004; Deng et al., 2012; Garcia-Angulo et al., 2008; Tobe et al., 2006). The target proteins of Nle effectors in host cells have started to be identified (Baruch et al., 2011a; Gao et al., 2009; Nadler et al., 2010; Newton et al., 2010; Pham et al., 2012; Royan et al., 2010; Sham et al., 2011; Vossenkamper et al., 2010; Wan et al., 2011; Yen et al., 2010); however, it remains largely unknown how Nle effectors interfere with cell signaling cascades and dampen the immune responses in host cells. The recognition of pathogens by host sensors activates multiple signaling pathways to induce inflammatory responses and eradicate the pathogens (Le Negrate, 2012). Among those, the NF-κB signaling pathway is crucial for host defense, as it orchestrates both innate and adaptive immune responses (Le Negrate, 2012). On the other hand, A/E bacteria, like other successful pathogens, have acquired sophisticated mechanisms to modulate host NF-κB signaling pathways (Clements et al., 2012; Lilic et al., 2003; Mulhern et al., 2009; O'Callaghan and Stebbins, 2010; Rahman and McFadden, 2006, 2011; Santoro et al., 2003). Not surprisingly, a handful of the Nle effector target proteins within host cells have been revealed to be NF-κB signaling molecules (Baruch et al., 2011a; Gao et al., 2009; Muhlen et al., 2011; Nadler et al., 2010; Newton et al., 2010; Pearson et al., 2011; Royan et al., 2010; Vossenkamper et al., 2010; Wan et al., 2011; Yen et al., 2010). Notably, however the molecular mechanisms through which each of these Nle effectors modulate NF-κB signaling have not been fully elucidated (Rahman and McFadden, 2011; Ruchaud-Sparagano et al., 2007).

Besides the well-defined Rel family proteins (RelA/p65, RelB, c-Rel, p50 and p52) (Hayden and Ghosh, 2004), RPS3 and Src-associated substrate during mitosis of 68kDa (Sam68) were recently identified as "specifier" components of NF-κB (Wan and Lenardo, 2009), where they modulate the promoter selectivity and transcriptional specificity of NF-κB (Fu et al., 2013; Wan et al., 2007). The

nuclear translocation and "specifier" function of RPS3 have been revealed to be tightly regulated by NF-κB signaling cascades (Wan et al., 2011). Specifically, RPS3 is found in the cytoplasmic p65-p50-IκBα inhibitory complex in resting cells (Wan et al., 2007). External stimuli activate the IκB kinase (IKK) complex, of which IKKβ phosphorylates IκBα resulting in its subsequent ubiquitination and degradation. IκBα removal unmasks a nuclear localization sequence (NLS), which allows nuclear import of p65 and p50 (Alkalay et al., 1995). Likewise, IKKβ phosphorylates RPS3 at serine 209 (Ser209), independently enhancing the RPS3-importin-α interaction for nuclear translocation. Once in the nucleus, RPS3 cooperates with p65 to target NF-κB to select promoters and to trans-activate those genes (Wan et al., 2011). Of note, the significance of RPS3/NF-κB signaling pathway has been highlighted in an increasing number of pathophysiological conditions (Cadera et al., 2009; Gao et al., 2009; Mokhtari et al., 2009; Sen et al., 2012; Wan et al., 2007; Wan et al., 2011), particularly in host proinflammatory transcription and immune responses against enteric pathogen infections (Gao et al., 2009; Wan et al., 2011). More specifically, the EHEC NleH1 effector inhibits the nuclear translocation of RPS3, but not p65, during NF-κB activation by tempering RPS3 Ser209 phosphorylation (Gao et al., 2009; Wan et al., 2011). As a consequence, NleH1 reduces the transcription of select, but not all, NF-κB target genes; most of the NleH1-attenuated RPS3/NF-κB-dependent genes encode proinflammatory cytokines/chemokines (Gao et al., 2009; Wan et al., 2011). In support of the critical role of RPS3 in the transcriptional selectivity of NF-κB genes, we recently demonstrated that modulating the RPS3/p65 interaction by ectopic expression of an N-terminal fragment (amino acids 21-186) of p65 attenuates RPS3 nuclear translocation, without affecting p65, thus selectively blocking a subset of specific NF-κB gene transcription (Wier et al., 2012).

NleC, a zinc-dependent protease effector conserved among A/E pathogens, was recently identified as one of the key effectors that dampen the innate immune response in host cells, particularly the production of inflammatory cytokines including interleukin-8 (IL-8) as well as others (Pearson et al., 2011; Sham et al., 2011; Yen et al., 2010). Mutagenesis of the consensus zinc

metalloprotease motif $_{183}$HEIIH$_{187}$ abrogates the proteolytic activity of NleC and cleavage of host target proteins (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Yen et al., 2010). Although p300 (Shames et al., 2011), IκB (Muhlen et al., 2011), and the NF-κB Rel proteins p65 and p50 (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Shames et al., 2011; Yen et al., 2010) have been reported as targets of NleC, it is largely understood that NleC cleaves and inactivates the NF-κB signaling pathway by primarily targeting the Rel proteins (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Yen et al., 2010), in line with the known critical role of NF-κB in the transcription of inflammatory cytokine genes (Vallabhapurapu and Karin, 2009). Previous studies have shown that ectopically expressed or T3SS-translocated NleC degrades p65, p50 and c-Rel, but not signal transducer and activator of transcription 1 (STAT1) or extracellular-signal-regulated kinases (ERKs), which indicates there may be cleavage specificity of NleC for the Rel subunits of NF-κB signaling pathway (Baruch et al., 2011b; Pearson et al., 2011). However, work by Yen *et al.* suggests that NleC could be specific for p65, as recombinant NleC could not digest p50 in cell lysates (Yen et al., 2010). Moreover, two cleavage site(s) on p65 by NleC have been identified as between proline 10 and alanine 11 (P10/A11) (Yen et al., 2010) or cysteine 38 and glutamic acid 39 (C38/E39) (Baruch et al., 2011b; Li et al., 2014; Turco and Sousa, 2014), although none of these studies ruled out the other cleavage site experimentally. The detailed mechanisms on how NleC specifically recognizes and cleaves p65 remain poorly understood. Moreover, even though recombinant proteins and ectopic expression of NleC in cell lines were employed in previous studies, only a very small percentage of p65 was shown to be cleaved by NleC with a large portion of full-length p65 still present within the cells (Baruch et al., 2011b; Yen et al., 2010). In contrast, it is puzzling that previous studies reported that NF-κB activity by luciferase assays and IL-8 production were markedly increased in HeLa cells infected with EPEC *ΔnleC* mutant, compared to wild-type EPEC (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010).

Here we reveal that the N-terminus of p65 is specifically recognized by NleC, which is required for the subsequent NleC-mediated cleavage. Infection of mice with a *C. rodentium* mutant strain lacking NleC (*ΔnleC)* augmented the transcription of several proinflammatory cytokine genes including *Cxcl1*, *Cxcl2*, *Il1b*, *Ifng*, and *Il22*, and triggered more immune cell infiltration in the colon, compared to wild-type *C. rodentium* inoculation. Moreover, NleC primarily cleaves p65 at C38/E39 during *C. rodentium* infection, and we show that the generated $p65^{1-38}$ fragment binds to RPS3 in NF-κB complexes and selectively retards the nuclear translocation of RPS3, but not p65. While only a small percentage of molecules are cleaved, the association between the $p65^{1-38}$ fragment and RPS3 that interferes with RPS3/p65 interaction-mediated transcription amplifies the impact of NleC cleaving p65. Therefore our results reveal a novel mechanism by which A/E pathogens selectively dampen RPS3 signaling and ensure inhibition of the RPS3/NF-κB-dependent inflammatory responses in host cells.

2.4 Results

2.4.1 NleC specifically cleaves p65, but not other NF-κB subunits

The T3SS effector NleC from various A/E pathogens has been proposed to dampen the NF-κB-mediated proinflammatory responses in host cells by functioning as a metalloprotease that cleaves NF-κB, albeit through poorly-defined mechanism(s) (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010). Moreover, Rel subunits p65 (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010), p50 (Muhlen et al., 2011), and c-Rel (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010) were proposed to be NleC target proteins. Alignment of the N-terminal sequences of both human and mouse Rel family proteins reveals that the best-characterized cleavage site, C38/E39 within the Rel homology domain, in p65 is also conserved among all human and mouse Rel proteins (Figure 2.1A

and Figure 2.2), indicating that NleC could potentially cleave all Rel proteins. To clarify which Rel protein(s) are NleC targets, we incubated whole cell lysates derived from HEK293T cells with recombinant NleC protein and examined the cleavage of endogenous Rel proteins using antibodies that specifically recognize the C-terminus of each Rel subunit. Consistent with previous studies (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010), a percentage of endogenous p65 was cleaved by NleC at the N-terminus thus generating a large C-terminal fragment (Figure 2.1B), whereas a catalytically inactive mutant of NleC, *i.e.* NleC (H117Y) with histidine 117 in the HExxH motif replaced by tyrosine (Yen et al., 2010), failed to do so (Figure 2.3). Moreover, NleC cleaved p65 to a similar extent in the presence and absence of tumor necrosis factor (TNF), a strong NF-κB stimulus (Figure 2.4), indicating that the NleC-mediated partial cleavage of p65 is not due to protection by other p65-binding proteins in the cytoplasm. In contrast to p65, the cleavage of other endogenous Rel proteins was not detectable, even when incubated with overwhelming amount (10 μg) of NleC recombinant protein (Figure 2.1B). Moreover, recombinant NleC cleaved the N-terminally FLAG-tagged p65 protein, with the tag allowing for better resolution of the small N-terminal fragment (Figure 2.1C). Of note, although recombinant NleC was able to cleave the ectopically expressed FLAG-tagged p50 protein, the product size marked by the N- and C-terminal FLAG tag indicates that cleavage occurs at the C-terminus of p50 rather than the conserved cysteine 62/glutamic acid 63 in the N-terminus (Figure 2.5), which corresponds to C38/E39 in p65. In addition, recombinant NleC failed to cleave ectopically expressed RPS3, which is a non-Rel subunit of NF-κB that confers the promoter selectivity and transcriptional specificity (Wan et al., 2007), in the recombinant protease cleavage assay (Figure 2.6). Therefore, NleC appears to selectively cleave p65, but not other NF-κB subunits.

2.4.2 The N-terminal 20 amino acids of p65 are essential for NleC-mediated cleavage

Notably the first 20 amino acids of p65 are unique to this protein and not conserved among the Rel proteins (Figure 2.1A and Figure 2.2), providing a clue to why NleC cleaves p65 specifically,

despite the fact that the C38/E39 cleavage site is shared by all other Rel subunits. Protease-substrate interaction-induced conformational changes are known to play an important role in the optimal cleavage of substrates by proteases (Sorimachi et al., 2012). We therefore hypothesized that the unique N-terminal sequence (a.a. 1-20) could be critical for the interaction between p65 and NleC, and be a prerequisite for NleC-mediated p65 cleavage. Using a library of C-terminal GFP-tagged p65 truncation constructs (Wan et al., 2007), we mapped the necessary region(s) of p65 for the NleC-conferred cleavage. Interestingly, recombinant NleC cleaved the $p65^{1-186}$ truncation as expected, whereas the $p65^{21-186}$ truncation harboring the C38/E39 cleavage site but missing the first 20 amino acids was not cleaved by NleC, even in the presence of an overwhelming amount (10 μg) of the recombinant protease (Figure 2.7A). To confirm this, we moved the GFP tag to the N-terminus of $p65^{21-186}$ truncation to increase resolution of the cleavage product and we were still unable to detect cleavage (Figure 2.8). These data suggest that the N-terminal 20 residues and C38/E39 cleavage site are both required for NleC-mediated p65 cleavage. In support of this notion, the full-length p65 containing both elements was cleaved by NleC, whereas no cleavage was detected in the $p65^{186-311}$ and $p65^{311-551}$ truncations as well as GFP vehicle control that do not contain either element (Figure 2.7A, 3A-3B and S6). Together these results demonstrate that the first 20 amino acids are essential for NleC-mediated cleavage to occur, thus providing a rational for the specificity of NleC for p65 rather than other Rel homology proteins, despite a shared Rel homology domain sequence.

2.4.3 The N-terminal 20 amino acids of p65 are required for NleC recognition

It has been widely acknowledged that the recognition of a substrate by a protease is critical for subsequent conformational changes to form a stabilized tetrahedral intermediate and to initiate optimal cleavage (Sorimachi et al., 2012). To examine whether the N-terminal 1-20 residues of p65 are required for NleC to recognize and bind p65, we incubated the whole cell lysates from HEK293T cells expressing full-length or truncated p65, with catalytically inactive NleC (H117Y) mutant or wild-type NleC at 4°C and conducted pull-down assays using nickel beads. NleC associated with full-

length p65, but did not interact with $p65^{311-551}$ (Figure 2.9), in line with the evidence that NleC cleaves full-length p65 rather than the truncated $p65^{311-551}$ (Figure 2.7A). Moreover, a significant interaction between NleC (H117Y) and $p65^{1-186}$ was detected, whereas there was little, if any, interaction between NleC (H117Y) and $p65^{21-186}$ (Figure 2.7B and Figure 2.9), suggesting that the N-terminal 20 amino acids could be the key targeting sequence for NleC to specifically recognize and bind to p65.

2.4.4 The major NleC cleavage site on p65 is between cysteine 38 and glutamic acid 39

Previous studies showed that C38/E39 is the NleC cleavage site in p65 (Baruch et al., 2011b; Li et al., 2014; Turco and Sousa, 2014), whereas P10/A11 was also reported as an NleC cleavage site (Yen et al., 2010). We therefore conducted recombinant NleC cleavage assays using lysates containing p65 N-terminally tagged with GFP, which allowed us to further examine the cleavage location(s) on the N-terminus of p65. In agreement with our previous results (Figure 2.7A), recombinant NleC cleaved p65 at the N-terminus generating two fragments that were N-terminally tagged with GFP; the cleaved products, which migrate at 37 kDa and 30 kDa, respectively, were verified using an antibody that specifically recognizes the N-terminus of p65 (Figure 2.10A-B). Of note, substantially more GFP-tagged $p65^{1-38}$ fragment was detected, indicating that NleC chiefly cleaves p65 at C38/E39. Therefore our results, in line with previous reports (Baruch et al., 2011b; Li et al., 2014; Turco and Sousa, 2014; Yen et al., 2010), demonstrate that the N-terminus of p65 harbors two NleC cleavage sites, P10/A11 and C38/E39, of which C38/E39 is the primary cleavage site whereas P10/A11 appears to be the secondary one (Figure 2.10C). In support of this notion, the resolved p65 crystal structure (Chen et al., 1998) reveals that the C38/E39 residues are located in a surface loop of the protein, which facilitates protease access (Figure 2.10D). Moreover, the NleC-generated $p65^{1-38}$ fragment is substantially reduced by an alanine substitution to C38/E39 (Figure 2.10E). However, the NleC-cleaved $p65^{1-10}$ fragment was also abolished by the C38A/E39A mutation. In contrast, the NleC-generated $p65^{1-38}$ fragment is less profoundly impacted by the alanine

substitution to P10, despite a complete inhibition of the NleC mediated cleavage of p65 at P10/A11 (Figure 2.10E). These results therefore strengthen our conclusion that C38/E39 is the major NleC cleavage site within p65. Moreover, lysates from normal HEK293T cells were subjected to the NleC cleavage assays to examine how recombinant NleC cleaves endogenous p65. As detected by an antibody specific for the p65 N-terminus, a p65 fragment migrating as approximately 10 kDa on SDS/PAGE gels was generated by NleC in a dose dependent fashion (Figure 2.10F). Because cleavage of p65 at P10/A11 would result in a fragment approximately 1 kDa in size that would likely be degraded, we therefore determined the cleaved p65 fragment is most likely the product of cleavage at C38/E39. Likewise, we observed a larger C-terminal fragment, migrating around 60 kDa on SDS/PAGE, using an antibody specific for the C-terminus of p65 (Figure 2.10G). Together, our results suggest that C38/E39 is the major NleC cleavage site on p65, which generates a detectable $p65^{1-38}$ fragment.

2.4.5 NleC cleaves p65 and generates the $p65^{1-38}$ fragment during C. rodentium *and EPEC infections*

To further examine the pathophysiological relevance of NleC-mediated p65 cleavage, we employed the EPEC and *C. rodentium* infection models. After 3-hour infection of wild-type *C. rodentium*, we detected the $p65^{1-10}$ and $p65^{1-38}$ fragments in HEK293T cells expressing GFP-tagged p65 (Figure 2.11A). The $p65^{1-10}$ and $p65^{1-38}$ cleavage was abolished in the cells infected with a *C. rodentium* mutant strain lacking NleC (*ΔnleC*) (Sham et al., 2011), compared to wild-type *C. rodentium* (Figure 2.11A). Moreover, the attenuated p65 cleavage was robustly restored in the cells infected with a *C. rodentium* mutant strain that lacks NleC but was complemented with a HA-NleC plasmid (*ΔnleC*/pHA-NleC) (Sham et al., 2011) (Figure 2.11A). Notably, we also observed the NleC-cleaved endogenous $p65^{1-38}$ fragment in Caco-2 cells, a human colon cancer cell line, and isolated mouse primary colon epithelial cells (CECs) infected by wild-type EPEC and *C. rodentium*, respectively (Figure 2.11B and 4C). Strikingly, the $p65^{1-38}$ fragments were greatly diminished in cells infected with *ΔnleC* mutant bacteria; whereas the p65 cleavage was rescued in cells infected with complemented

ΔnleC/pHA-NleC strains (Figure 2.11B and C), suggesting that NleC cleaves p65 thus generating the $p65^{1-38}$ fragment during EPEC and *C. rodentium* infections in cell culture. In contrast to p65, the cleavage of p50 in Caco-2 cells and mouse CECs infected by EPEC and *C. rodentium*, respectively, was not detectable (Figure 2.12), further supporting that NleC specifically cleaves p65 among NF-κB subunits. To examine the possibility that the abolished p65 cleavage in CECs infected with *ΔnleC C. rodentium* could be due to defective attachment to host cells, in comparison to wild-type and complemented strains, we measured the attachment of variant *C. rodentium* strains to CECs during infection. As shown in Figure 2.11E, wild-type, *ΔnleC*, and *ΔnleC*/pHA-NleC *C. rodentium* attached to CECs in a similar pattern. Moreover, as assayed by enumeration of CEC-attached bacteria and immunoblot for marker proteins in mouse CECs (heat shock protein, Hsp90) and *C. rodentium* (lipopolysaccharides, LPS), the amount of *ΔnleC* and *ΔnleC*/pHA-NleC *C. rodentium* that attaches to CECs during infection was equal if not higher than the wild-type bacteria (Figure 2.11D and F). These results rule out the possibility that NleC deletion affects the interaction between *C. rodentium* and CECs, and suggest that NleC executes the p65 cleavage during EPEC and *C. rodentium* infections, generating a small amount of $p65^{1-38}$ fragment and leaving most p65 intact.

2.4.6 *NleC affects host immune responses in mice infected by* C. rodentium

Infection of *C. rodentium* in mice is known to cause colonic epithelial damage by acute inflammatory responses (Mundy et al., 2005), therefore we examined the impact of NleC on colonic inflammatory response in mice inoculated with variant strains of *C. rodentium*. As we reported previously (Sham et al., 2011), the colonization of wild-type and *ΔnleC* mutant *C. rodentium* in the colon of infected mice was comparable at day 8 and day 10 (Figure 2.13A and Figure 2.14, respectively). In line with previous reports that NleC-mediated p65 cleavage plays a critical role in dampening the NF-κB signaling pathway and suppressing proinflammatory gene expression in EPEC-infected cells (Pearson et al., 2011; Yen et al., 2010), we observed robust transcription of known NF-κB target genes *Cxcl1* and *Cxcl2* in the colon tissues removed from mice infected with

ΔnleC C. rodentium, compared to wild-type bacterium at day 14 (Figure 2.13B). We also detected significantly elevated levels of additional NF-κB target genes *Ifng, Il1b,* and *Il22*, thus highlighting the key function of NleC-mediated p65 cleavage in interfering with host NF-κB gene transcription (Figure 2.13B). Consistent with the colonic expression of proinflammatory cytokine/chemokine genes, the amount of infiltrated $CD11b^+$ myeloid cells in the colon was increased by two fold in mice orally inoculated with *ΔnleC C. rodentium*, compared to that from mice infected with wild-type bacterium (Figure 2.13C-D). As expected, wild-type *C. rodentium*-infected mice developed severe clinical symptoms characterized by crypt elongation, thickening of the mucosal surface, and goblet cell depletion in histological staining, in comparison to phosphate-buffered saline (PBS) inoculated animals (Figure 2.13E-F). By contrast, infection with *ΔnleC C. rodentium* induced more severe damage to colon epithelia and general enlargement of the colonic tissue (Figure 2.13E-F). Our results therefore demonstrate that the p65 cleavage by NleC during *C. rodentium* infection has a dramatic effect on NF-κB gene transcription and inflammatory response in the infected animals.

2.4.7 The NleC-cleaved $p65^{1-38}$ interacts with RPS3

It is noteworthy that NleC cleaved only a small percentage of p65 during *C. rodentium* infection (Figure 2.11A-C) and even in the presence of an overwhelming amount (10 μg) of recombinant protease (Figure 2.1B, Figure 2.7A, Figure 2.10A-B, F-G). In particular, the dramatic effect of NleC on the proinflammatory cytokine production and NF-κB activity was proposed to be mediated by the cleavage of p65 by NleC (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Shames et al., 2011; Yen et al., 2010); however, the large amount of full-length p65 resistant to NleC cleavage makes it difficult to explain the remarkable impact of NleC on dampening host NF-κB signaling and inflammatory response. Of note, RPS3, a non-Rel subunit of NF-κB, was revealed to confer the promoter selectivity and transcriptional specificity of NF-κB (Wan et al., 2007; Wan and Lenardo, 2010), in particular the RPS3/NF-κB-mediated transcription of a subset of proinflammatory genes is critical for host defense against A/E pathogens (Gao et al.,

2009; Pham et al., 2012; Wan et al., 2011). Moreover, our previous studies showed that interrupting the subcellular localization and function of RPS3 by small interfering RNA (siRNA) (Wan et al., 2007), bacterial effectors (Gao et al., 2009; Pham et al., 2012; Wan et al., 2011), and ectopic expression of an N-terminal truncated $p65^{21-186}$ fragment (Wier et al., 2012), are able to selectively block NF-κB target gene transcription, without affecting the nuclear translocation of p65. We therefore hypothesized that the NleC-cleaved $p65^{1-38}$ product would execute a similar function as the $p65^{21-186}$ fragment (Wier et al., 2012), which interferes with RPS3 signaling. To test this hypothesis, we examined the ability of GFP-tagged $p65^{1-38}$ and $p65^{39-551}$ truncated proteins to alter NF-κB signaling in cultured cells. As demonstrated by subcellular fractionation, both $p65^{1-38}$ and $p65^{39-551}$ truncated proteins were primarily located in the cytoplasm of transfected HEK293T cells, even following 30-min TNF treatment (Figure 2.15A). This result is in agreement with previous reports that NleC inactivates p65 in the cytoplasm (Baruch et al., 2011b; Yen et al., 2010), and suggests that the NleC-cleaved products would mainly interfere with cytoplasmic NF-κB signaling in host cells. We further examined the interaction between RPS3 and the GFP-tagged $p65^{1-38}$ and $p65^{39-551}$ fragments by immunoprecipitation. The RPS3-$p65^{1-38}$ interaction was comparable to, if not even stronger than, that of RPS3 and full-length p65, whereas the association between RPS3 and the $p65^{39-551}$ fragment was barely detectable (Figure 2.15B). Moreover, the $p65^{1-38}$ truncated protein was substantially enriched in the GST-RPS3 pulldown sample, compared to the GST vehicle control (Figure 2.16), which independently verifies the interaction between $p65^{1-38}$ and RPS3. Our results therefore suggest that the NleC-cleaved $p65^{1-38}$ product, rather than the $p65^{39-551}$ fragment, of p65 is able to interact with the NF-κB non-Rel subunit RPS3.

2.4.8 The $p65^{1-38}$ product selectively attenuates the nuclear translocation of RPS3

Stimuli-triggered translocation from the cytoplasm to the nucleus is a prerequisite for RPS3 to facilitate NF-κB binding and transactivation of specific target genes (Wan and Lenardo, 2009, 2010). We recently showed that ectopic expression of $p65^{21-186}$ truncated protein competed RPS3 off

endogenous full-length p65, thereby interfering with the nuclear translocation of RPS3 during the NF-κB response (Wier et al., 2012). Ectopic expression of the $p65^{1-38}$ fragment, compared to the GFP control, did not alter TNF-stimulated IκBα degradation (Figure 2.15C) and p65 nuclear translocation (Figure 2.15D). By contrast, overexpression of $p65^{1-38}$ fragment remarkably attenuated TNF-triggered nuclear translocation of RPS3, which was induced normally in the GFP-expressing cells (Figure 2.15D). To further assess the impact of $p65^{1-38}$ fragment on NF-κB activation, we examined the expression of an Ig-κB site-driven luciferase reporter gene, which was previously shown to be RPS3-dependent (Wan et al., 2007; Wan et al., 2011; Wier et al., 2012), in HEK293T cells expressing the $p65^{1-38}$ fragment. Indeed, in comparison to the GFP vehicle control, ectopic expression of the $p65^{1-38}$ fragment attenuated NF-κB reporter luciferase expression in a dose dependent manner (Figure 2.15E). These results suggest that the $p65^{1-38}$ fragment is capable of interfering with the endogenous p65/RPS3 interaction and selectively attenuating the nuclear translocation of RPS3 rather than affecting other branches of NF-κB signaling (Figure 2.17), which provides a novel mechanism for A/E pathogens to specifically modulate host NF-κB-mediated gene transcription and inflammatory responses.

2.5 Discussion

The NF-κB signaling pathway orchestrates both innate and adaptive immune responses in host cells, thereby executing an important function in host defense to a variety of pathogens (Le Negrate, 2012). That said, the mechanisms controlling promoter selectivity and transcriptional specificity of NF-κB genes in acute pathogen-host interactions remain obscure. Beyond the well-characterized NF-κB Rel subunits, we recently identified two non-Rel subunits, *i.e.* RPS3 and Sam68, in the NF-κB DNA binding complexes that confer distinct transcriptional specificity of NF-κB (Wan and Lenardo, 2009, 2010). The RPS3- and Sam68-conferred NF-κB activation model suggests that the Rel subunits are required, but not the sole determinants, for the activation of NF-κB target genes. In contrast, the synergistic interactions between Rel and non-Rel subunits at the promoters are

critical for the transactivation of certain NF-κB target genes (Wan and Lenardo, 2009, 2010). An increasing number of studies (Cadera et al., 2009; Gao et al., 2009; Mokhtari et al., 2009; Sen et al., 2012; Wan et al., 2007; Wan et al., 2011) suggest that the RPS3/NF-κB signaling pathway is vital in the host proinflammatory transcription and immune responses against infection by A/E pathogens (Gao et al., 2009; Wan et al., 2011). Specifically, the T3SS effector NleH1 from EPEC, EHEC, and *C. rodentium* attenuates the nuclear translocation of RPS3, but not p65, during NF-κB activation by inhibiting the IKKβ-mediated Ser209 phosphorylation of RPS3 (Gao et al., 2009; Wan et al., 2011). This leads to reduced transcription of select, but not all, NF-κB target genes, most of which are RPS3/NF-κB-dependent proinflammatory cytokine genes (Gao et al., 2009; Wan et al., 2011). In this work, we show that the T3SS effector protease NleC predominantly cleaves p65 at C38/E39. The NleC cleavage-generated $p65^{1-38}$ fragment interferes with the RPS3/p65 interaction in the cytoplasm, resulting in the attenuated nuclear translocation of RPS3, without affecting p65 nuclear translocation. Similar to what we have shown previously (Gao et al., 2009; Wan et al., 2007; Wan et al., 2011; Wier et al., 2012), cytoplasmic sequestering of RPS3 prevents the transactivation of the RPS3/p65-dependent proinflammatory cytokine genes and the infiltration of inflammatory immune cells to infected tissue. Of note, NleC cleaves a small percentage of p65 in *C. rodentium*-infected mouse CECs. In contrast, NleC cleavage has a substantial effect on proinflammatory cytokine gene transcription, as evidenced by infections using the EPEC and *C. rodentium* genetic mutants with disruptions in the NleC gene, compared to wild-type strains. We are aware that NleC, as a metalloprotease, cleaves other host proteins beyond p65, such as p300 (Shames et al., 2011), p38 (Sham et al., 2011), and others, which could also result in the observed striking effects of NleC on colonic cytokine gene expression and inflammatory responses in infected mice. However, our proposed "amplifying" mechanism through the RPS3-conferred NF-κB specific transcription provides a novel explanation for the disconnect regarding the extent of NleC-mediated NF-κB cleavage and the impact of NF-κB transactivation by such cleavage. In conjunction with our previous studies showing that NleH1 attenuates RPS3 Ser209 phosphorylation (Gao et al., 2009; Wan et al.,

2011), our results highlight the critical role of the RPS3/NF-κB signaling pathway in the host immune response to A/E pathogen infections. Although NleC and NleH1 are both secreted by the A/E pathogen T3SS, the kinetics and amount of NleC and NleH1 within host cells during infection remains largely unknown. It has been well documented that certain successful pathogens have acquired sophisticated mechanisms to directly interfere with host NF-κB signaling through regulating or mimicking host proteins to their own advantage, and these co-opted strategies can operate at multiple levels of the sequential process of NF-κB signaling (Baruch et al., 2011a; Gao et al., 2009; Muhlen et al., 2011; Nadler et al., 2010; Newton et al., 2010; Pearson et al., 2011; Royan et al., 2010; Vossenkamper et al., 2010; Wan et al., 2011; Yen et al., 2010). We therefore speculate that A/E pathogens utilize NleC and NleH1 to simultaneously (or sequentially) interfere with the RPS3/NF-κB signaling pathway through distinct strategies, therefore ensuring with this redundancy that the NF-κB signaling and inflammatory responses are dampened in host cells. Notably, the RPS3-p65 branch of NF-κB signaling has been associated with transcription of anti-apoptotic genes (Sen et al., 2012) and inflammatory genes (Gao et al., 2009; Wan et al., 2011) therefore selectively targeting this arm could prove advantageous to the pathogen. Given that the spatial and temporal coordination of effectors injected into host cells remains unclear (Shames and Finlay, 2012; Wong et al., 2011), it is intriguing to consider that manipulating inflammatory gene transcription would be valuable early during infection and ensuring apoptosis would be an escape strategy allowing the pathogen to spread to the next host. While it has been documented that a relatively low amount of NleC is delivered into host cells during infection in cultured cells (Deng et al., 2012), the timing, longevity, and activity of the effectors within the host cell during an *in vivo* animal infection remains elusive. Greater resolution of the timing and amount of NleC's introduction into the host cell and interaction with p65 is still needed.

The most abundant species among the NF-κB complexes in cells consists of p65, p50, and other proteins (Wan et al., 2007). Moreover, p65 possesses a transactivation domain (TAD) that is

essential to recruit general transcriptional machinery to transcribe target genes, whereas p50 lacks the TAD domain thereby normally suppressing gene expression (Wan and Lenardo, 2009). These features make p65 unique within the NF-κB Rel subunits and a likely target for pathogen effectors. Our results show that NleC cleaves p65 more efficiently than other Rel family proteins and non-Rel subunits, RPS3 and Sam68. In further support of this notion, the N-terminal 20 residues of p65, which are not conserved among other Rel family proteins, play a critical role in the recognition and cleavage of p65 by NleC during A/E pathogen infection. Therefore it is not surprising that p65, the most important NF-κB Rel subunit, is a major target for a wealth of pathogens, allowing them to interfere with the NF-κB signaling pathway in host cells (Baruch et al., 2011b; Muhlen et al., 2011; Pearson et al., 2011; Sham et al., 2011; Yen et al., 2010). Indeed, previous studies showed that p65 was cleaved by a myriad of pathogen encoded proteases (Cameron et al., 2004; Christian et al., 2010; Coiras et al., 2008; Doyle et al., 2011; Neznanov et al., 2005; Silva et al., 2013), although the direct consequence of cleaving p65 on NF-κB signaling has not been extensively studied. For instance, *Chlamydia trachomatis*, a gram-negative bacterium that causes urethritis, cervicitis, and other diseases, encodes a PDZ containing tail-specific protease (Lad et al., 2007b). The enzyme was proposed to suppress host NF-κB activity by cleaving p65 and generating two p65 fragments (approximately 40 kDa and 25 KDa, respectively) (Lad et al., 2007a). Moreover, the A-B toxin metalloprotease encoded by the fish pathogen *Photobacterium damselae* piscicida was recently shown to cleave p65 at C38/E39, similar to NleC (Silva et al., 2013). It would be interesting to examine if p65 fragment interference is a previously unrecognized but possibly widespread mechanism of virulence for abrogating host NF-κB signaling, especially when pathogen encoded proteases are unable to cleave the majority or entire cellular source of p65. By selectively blocking NF-κB "specifiers", as we demonstrated here for RPS3, pathogens could more acutely manipulate the host environment by regulating the timing and abundance of injected effectors, allowing collections of genes to be turned on and off to their advantage.

2.6 Material and Methods

Ethics statement. All animal experiments were performed according to protocol number MO13-H349, approved by the Johns Hopkins University's Animal Care and Use Committee and in direct accordance with the NIH guidelines for housing and care of laboratory animals.

Cell line, antibodies, and plasmids. HEK293T and Caco-2 cells (ATCC, Manassas, VA) were cultured in DMEM medium containing 10% fetal calf serum, 2 M glutamine, 100 U ml^{-1} penicillin, and 100 U ml^{-1} streptomycin. Antibodies used were: p65 (C-terminus, C-20, sc-372), p65 (N-terminus, F-6, sc-8008x), p50 (NLS, sc-114), c-Rel (C, sc-71) from Santa Cruz Biotechnology (Dallas, TX); β-actin (AC-15, A5441) and FLAG (M2, F1804) from Sigma-Aldrich (St. Louis, MO); PARP-1 (46D11, 9532) from Cell Signaling Technology (Danvers, MA); GFP (7.1 and 13.1, 11814460001) from Roche Applied Science (Indianapolis, IN); CD11b (M1/70, 101202) from BioLegend (San Diego, CA); Hsp90 (610418) from BD Biosciences (San Jose, CA); *E. coli* O152 LPS (81449) from Statens Serum Institut (Copenhagen, Denmark); RPS3 as previously described (Wan et al., 2007). Tumor necrosis factor (TNF) was purchased from R&D System (Minneapolis, MN). The plasmids His-NleC (Pearson et al., 2011), FLAG-p50, p50-FLAG (Dooher et al., 2011), GFP-tagged full-length p65, $p65^{1-186}$, $p65^{21-186}$, $p65^{1-311}$, $p65^{186-311}$, and $p65^{311-551}$ (Wan et al., 2007) were previously described. The GFP-$p65^{1-38}$ and GFP-$p65^{39-551}$ were generated by inserting the appropriate fragments into the pEGFP-N1 vector (Clontech Laboratories, Mountain View, CA) using the InFusion Cloning System (Clontech Laboratories). The His-NleC (H117Y) and GFP-tagged p65 (C38A/E39A) and p65 (P10A) mutants were generated by site-directed mutagenesis using the Quick Change Kit (Stratagene, La Jolla, CA) with appropriate primers. All the plasmids were verified by DNA sequencing.

Transient transfection. DNA constructs were transfected into HEK293T cells using the TurboFect *in vitro* transfection reagent (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions, as described previously (Wier et al., 2012).

NleC protease digestion assays. Overnight cultures of BL21 (pET-NleC) were induced with1 mM IPTG and His-NleC proteins were purified by nickel affinity chromatography. The NleC protease digestion was conducted as previously described (Pearson et al., 2011). Briefly, cells were collected and lysed on ice with 0.4 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate, 1 × complete protease inhibitor cocktail [Roche Applied Science]) for 30 min. After centrifuge at 10,000 × *g* at 4°C for 10 min, 200 μl of supernatant was removed to a separate tube and incubated with indicated amount of His-NleC protein at 37°C for 3 h.

Nickel bead and GST pull-down assays. For the interactions between NleC and indicated proteins, the 200 μl of supernatant mixed with 1 μg of His-NleC protein were subjected to pull-down assays by adding 30 μl of Nickel beads (Qiagen, Germantown, MD), and rotating for 30 min at 4°C. The GST pulldown assays were conducted as previously described (Fu et al., 2013). The pull-down proteins were washed at least four times with cold lysis buffer followed by separation with SDS-PAGE.

Isolation of primary colon epithelial cells. Colon epithelial cells (CECs) were isolated from C57BL/6J mice as previously described (Flint et al., 1991). Briefly, after euthanizing mice, the entire colon was removed under aseptic conditions and washed twice with ice-cold PBS. After dividing the colon into 2–3 mm long fragments and transferring them into chelating buffer (27 mM trisodium cirtcrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 0.5 mM DTT, 55 mM D-sorbitol, 44 mM sucrose, 6 mM EDTA, 5 mM EGTA [pH 7.3]) for 45 min at 4°C, CECs were then dislodged by repeated vigorous shaking. Tissue debris was removed by a 70-μm cell strainer (Fisher Scientific, Suwanee, GA) and CECs were harvested by centrifugation at 4°C. The viability of CECs was confirmed by trypan blue staining and isolated CECs were cultured at 37°C for 1 h for recovery, followed by infection.

***Citrobacter rodentium* and EPEC growth conditions and infection in cultured cells.** Wild-type *C. rodentium* (DBS 100) and EPEC (E2348/69), as well as the NleC deletion mutant (*ΔnleC*) and the HA-NleC complemented (*ΔnleC*/pHA-NleC) strains (Sham et al., 2011) were grown from single colonies on Luria-Bertani (LB) plates in LB broth at 37°C overnight with shaking. Infection of EPEC in Caco-2 cells was performed as previously described (Sham et al., 2011). Prior to infection experiments, *C. rodentium* was washed with ice-cold PBS and resuspended in pre-warmed corresponding media. Bacteria concentration was measured by absorbance at optical density 600, followed by a serial dilution and seeding on a MacConkey agar plate (VWR, Radnor, PA) to confirm the administered colony-forming units (CFU). The HEK293T cells and isolated CECs were infected with the indicated strains of *C. rodentium* at a multiplicity of infection (MOI) of 100 for 3 h, as described previously (Sham et al., 2011). Cells were counted using a hemacytometer prior to each experiment and equal numbers of cells (1-2 × 10^6) were aliquoted into each infection condition. To determine bacterial attachment to CECs infected in suspension, cells and bacteria were passed through a Percoll gradient (40% and 60%) and CECs were collected from the top of half of the 40% gradients and overlay (Childs and Gibbons, 1988; Colombo et al., 2006; Izhar et al., 1982). Cells were washed with PBS and placed on cover slips or lysed for immunofluorescence staining or immunoblot.

Immunofluorescence staining in colon epithelial cells. Post *C. rodentium* infection, mouse CECs were spun down to Poly-L-Lysine-coated coverslips, fixed with 4% PFA, and stained with appropriate primary antibodies and fluorescence dye-conjugated second antibodies. Following staining of nuclei with 1 μg ml^{-1} of DAPI (Sigma-Aldrich), coverslips were mounted onto slides using Fluoro-gel with Tris Buffer (Electron Microscopy Sciences, Hatfield, PA) and examined using an Axio Observer fluorescence microscope (Zeiss, Oberkochen, Germany). The numbers of *C. rodentium* that attached to mouse CECs were quantified using ImageJ software (NIH, Bethesda, MD) and normalized to cell perimeter.

***Citrobacter rodentium* infection in mice.** Male C57BL/6 mice (6 to 8 weeks) purchased from the Jackson Laboratory (Bar Harbor, ME) were maintained in a specific pathogen-free facility and fed autoclaved food and water *ad libitum*. Food was withheld from the mice for 6-8 hours before they were orally inoculated with 200 μl of PBS containing 2×10^9 CFU of wild-type or *ΔnleC* mutant *C. rodentium* or PBS alone, and euthanized at the indicated time points post infection.

Colon tissue collection, histology, and immunofluorescence staining. After euthanizing mice, their colons were removed under aseptic conditions, washed once with ice-cold PBS, and the terminal 0.5-cm piece of the colon was frozen in optimal cutting temperature (O.C.T.) media (Tissue-Tek, Elkhart, IN) or incubated overnight in 4% PFA. 5-micron frozen sections were cut using a Microm HM 550 Cryostat (Thermo Scientific), collected on coated slides and processed for immunofluorescence staining. Frozen sections were fixed in paraformaldehyde, washed with PBS, and blocked with appropriate sera in PBS. After incubating with appropriate antibodies, sections were washed and incubated with fluorescence dye-conjugated second antibodies and 1 μg ml^{-1} of DAPI (Sigma-Aldrich). Stained sections were washed and mounted under a coverslip using Fluoro-gel with Tris Buffer (Electron Microscopy Sciences). For histological analysis, the colon tissue was embedded in paraffin and 5-micron sections were cut, collected on coated slides and processed for Hematoxylin and Eosin (H&E) staining. Stained sections were examined using an Axio Observer fluorescence microscope (Zeiss). Histopathology scores were determined in a blinded fashion using the following criteria as previously described by Qualls et al. (Qualls et al., 2006): 0, Normal tissue; Grade 1, mild inflammation was present containing mostly mononuclear cell infiltrate and little damage to the epithelia; Grade 2, inflammation greater than Grade 1 with mononuclear and polymorphonuclear infiltrate, mucin and Goblet cell depletion, and epithelium beginning to detach from basement membrane; Grade 3, inflammation and cellular infiltrate is greater than Grade 2 with cellular infiltrates reaching the submucosa, greater Goblet cell depletion, and greater epithelial disruption; Grade 4, severe inflammation containing mostly neutrophils, completely detached epithelium, and crypt destruction.

Quantitative real-time PCR. Total RNA was isolated from colon tissues using Trizol reagent (Life Technologies) and treated with the TURBO DNA-free Kit (Life Technologies) to remove residual genomic DNA. cDNA was synthesized using qScript cDNA SuperMix Kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Gene specific products were amplified using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) with the following primers:

Cxcl1-f, 5'-TGCACCCAAACCGAAGTCAT-3';

Cxcl1-r, 5'-TTGTCAGAAGCCAGCGTTCAC-3';

Cxcl2-f, 5'-CCTGCCAAGGGTTGACTTCA-3';

Cxcl2-r, 5'-TTCTGTCTGGGCGCAGTG-3';

Ifng-f, 5'-ATGAACGCTACACACTGCATC-3';

Ifng-r, 5'-CCATCCTTTTGCCAGTTCCTC-3';

Il1b-f, 5'-GAAATGCCACCTTTTGACAGTG-3';

Il1b-r, 5'-CTGGATGCTCTCATCAGGACA-3';

Il22-f, 5'-CAGAGGTAGACTTGATAACCAC-3';

Il22-r, 5'-GGTTATGGAAATGAAGTTACATAAGC-3'.

Subcellular fractionation. Subcellular fractionation was performed by differential centrifugation as previously described (Fu et al., 2013). Briefly, cells were resuspended in Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM DTT, 0.4 % NP-40, 0.5 mM PMSF, 1 × complete protease inhibitor cocktail [Roche Applied Science]) at 4°C for 5 min. Lysates were centrifuged at 4°C, 500 × *g* for 3 min, and supernatants were collected as cytosolic fractions. Pellets

were incubated in Buffer C (20 mM HEPES [pH 7.9], 420 mM NaCl, 1.5 mM $MgCl_2$, 25% glycerol, 0.5 mM PMSF, 0.2 mM EDTA, 0.5 mM DTT, 1 × complete protease inhibitor cocktail) at 4°C for 10 min, followed by a centrifuge at 4°C, 13,000 × *g* for 10 min. Supernatants were collected as nuclear fractions.

Immunoprecipitation and immunoblot. The cells were harvested and lysed on ice with 0.4 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate, 1 × complete protease inhibitor cocktail) for 10 min. The lysates were centrifuged at 10,000 × *g* at 4°C for 10 min. The protein-normalized lysates were subjected to immunoprecipitation by adding 10 mg ml^{-1} of the appropriate antibody, 30 μl of protein G-agarose (Roche Applied Science), and rotating for more than 2 h in the cold room. The precipitates were washed at least four times with cold lysis buffer followed by a separation by SDS-PAGE under reduced and denaturing conditions. The resolved protein bands were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA), probed as described previously (Fu et al., 2013), developed by the Super Signaling system (Thermo Scientific) according to the manufacturer's instructions, and imaged using a FluorChem E System (Protein Simple, Santa Clara, CA).

Luciferase reporter gene assays. Luciferase reporter gene assays were performed as previously described (Wan et al., 2007; Wan et al., 2011; Wier et al., 2012). Briefly, cells were cotransfected with 5 × Ig κB site-driven firefly luciferase constructs and the Renilla luciferase pTKRL plasmid (ratio 10:1), together with appropriate plasmids. Cells were cultured for 18 hours, stimulated in triplicate, and analyzed using the Dual-Luciferase Kit (Promega, Madison, WI).

Statistical analysis. All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). The difference between treated and control groups were examined by unpaired Student's *t* tests. Standard errors of means (s.e.m.) were plotted in graphs. n.s.

means non-significant difference and significant differences were considered * at $p < 0.05$; ** at $p < 0.01$; and *** at $p < 0.001$.

2.7 Acknowledgements

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2.8 Figures

Figure 2.1 NleC specifically cleaves p65/RelA among the NF-κB Rel family proteins.

A. Sequence alignment of the human Rel family proteins. The conserved Cys/Glu residues in their respective Rel domains and the unique N-terminal 20 residues in p65/RelA are highlighted. The numbers at right show the position in the amino-acid sequence of the last residues depicted. B. Whole cell lysates derived from HEK293T cells were incubated with indicated amount of His-NleC recombinant protein, followed by SDS/PAGE separation and immunoblotted for indicated proteins with antibodies specifically recognizing the C-termini of p65, p50 and c-Rel, respectively. The full-length p65 and cleaved p65 C-terminal fragment are indicated by filled and open triangles, respectively. C. Whole cell lysates derived from HEK293T cells expressing N-terminally FLAG-tagged p65 were incubated with indicated amount of His-NleC recombinant protein. The cleavage of FLAG-tagged p65 was immunoblotted with anti-FLAG antibody, following SDS/PAGE separation. The NleC cleavage site in p65 is indicated by a red triangle.

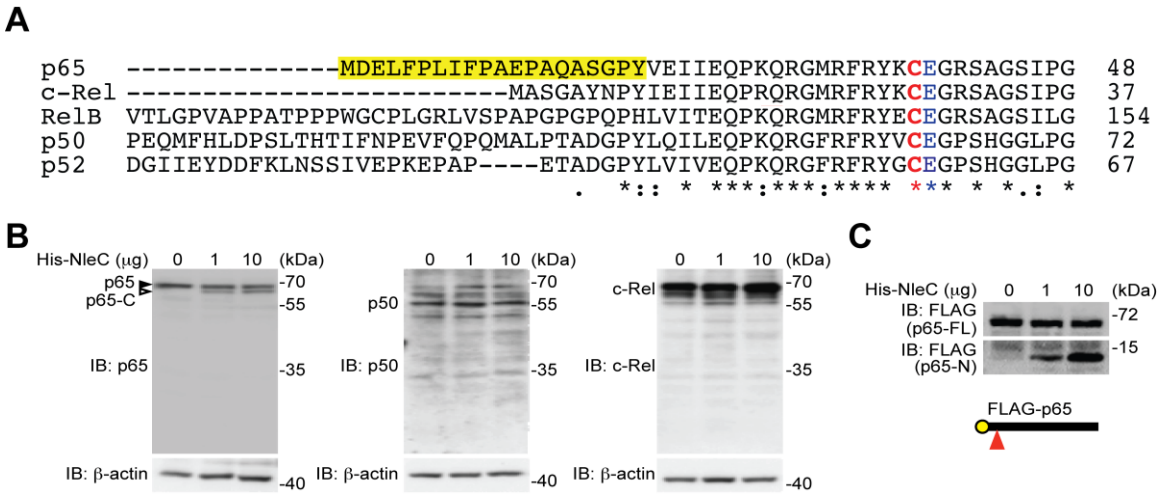

Figure 2.2 The first 20 amino acids of the mouse RelA are unique

The conserved Cys/Glu residues in their respective Rel domains in the mouse and the unique N-terminal 20 residues in p65/RelA are highlighted. The numbers at right show the position in the amino-acid sequence of the last residues depicted.

```
p65             --------------MDDLFPLIFPSEPAQASGPYVEIIEQPKQRGMRFRYKCEGRSAGSIPG 48
c-Rel           -------------------------MASSGYNPYVEIIEQPRQRGMRFRYKCEGRSAGSIPG 37
RelB            SVTLGPAAPPPPATPSWSCTLGRLVSPGPCPRPYLVITEQPKQRGMRFRYECEGRSAGSILG 132
p50             GTGQMFHLNTALTHSIFNAELYSPEIPLSTDGPYLQILEQPKQRGFRFRYVCEGPSHGGLPG 69
p52             LDGIPEYDDFEFSPSIVEPKDPAPET---ADGPYLVIVEQPKQRGFRFRYGCEGPSHGGLPG 67
                                             **: * ***:***:**** *** * *.: *
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Figure 2.3 The protease-dead NleC mutant is unable to cleave p65

Whole cell lysates derived from HEK293T cells were incubated with the indicated amount of wild-type (WT) or catalytically inactive H117Y mutant His-NleC recombinant protein, followed by SDS/PAGE separation and immunoblotted for indicated proteins. The full-length p65 and cleaved p65 C-terminal fragment are indicated by filled and open triangles, respectively.

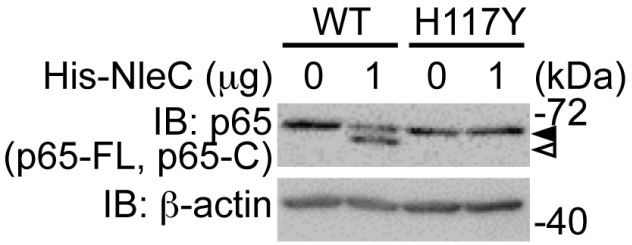


Figure 2.4 TNF stimulation does not alter NleC-mediated p65 cleavage

A-B. HEK293T cells (A) and HEK293T cells expressing N-terminally GFP-tagged p65 (B) were treated with 50 ng ml^{-1} of TNF for 30 min or the PBS vehicle control. Whole cell lysates were derived and incubated with the indicated amount of His-NleC recombinant protein, followed by SDS/PAGE separation and immunoblotted for indicated proteins. The full-length p65 and cleaved p65 C-terminal fragment are indicated by filled and open triangles, respectively.

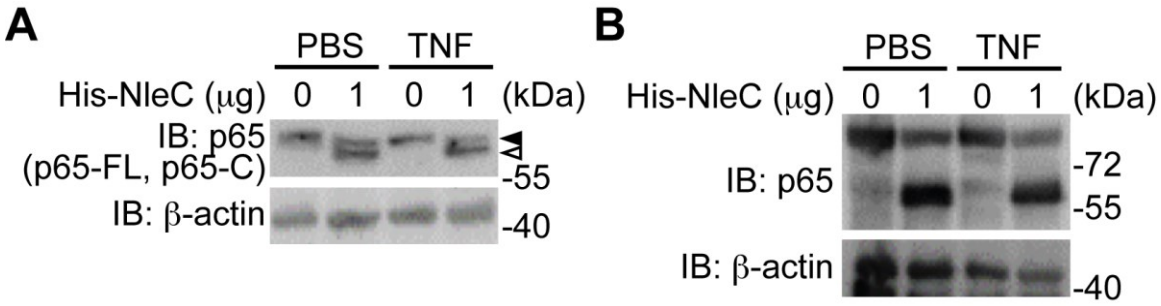


Figure 2.5 NleC-mediated cleavage of p50 occurs at the C-terminal in overexpression conditions

Whole cell lysates derived from HEK293T cells expressing N- and C-terminally FLAG-tagged p50 were incubated with the indicated amount of His-NleC recombinant protein. The cleavage of FLAG-tagged p50 was immunoblotted with anti-FLAG antibody, following SDS/PAGE separation. The NleC cleavage sites in p50 are indicated by red triangles.

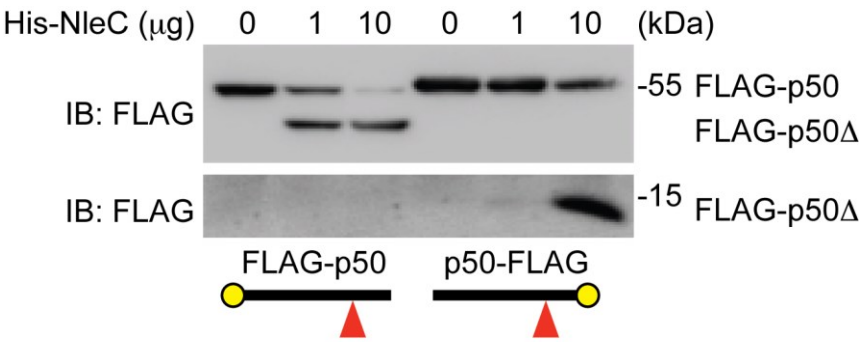


Figure 2.6 RPS3 is not a substrate of NleC

Whole cell lysates derived from HEK293T cells expressing FLAG-tagged RPS3 were incubated with indicated amount of His-NleC recombinant protein. The cleavage of FLAG-tagged RPS3 was immunoblotted with anti-FLAG antibody, following SDS/PAGE separation.

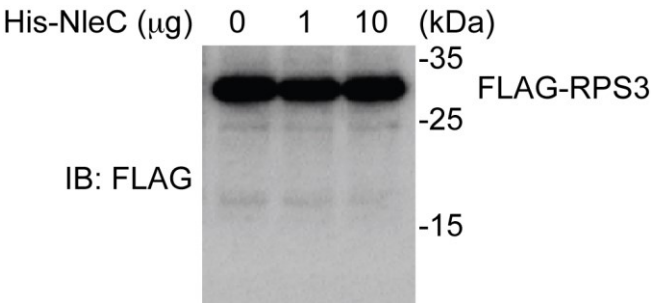


Figure 2.7 The N-terminal 20 amino acids of p65 are required for NleC to bind and cleave p65.

A. Various truncations of C-terminal GFP-tagged p65, as illustrated at the bottom, were transfected into HEK293T cells. Whole cell lysates were subjected to the His-NleC cleavage assay, and immunoblotted with anti-GFP antibody for NleC-cleaved fragments. The GFP-tagged p651-186 protein and cleaved fragment were labeled by filled and open triangles, respectively, and the NleC cleavage sites in p65 were indicated below. B. Whole cell lysates derived from HEK293T cells expressing GFP or the indicated GFP-tagged p65 proteins were incubated with the catalytic mutant His-NleC (H117Y) at 4°C. Nickel beads were added to pull-down His-NleC and associated proteins. Samples were separated by SDS/PAGE, followed by immunoblot for indicated proteins.

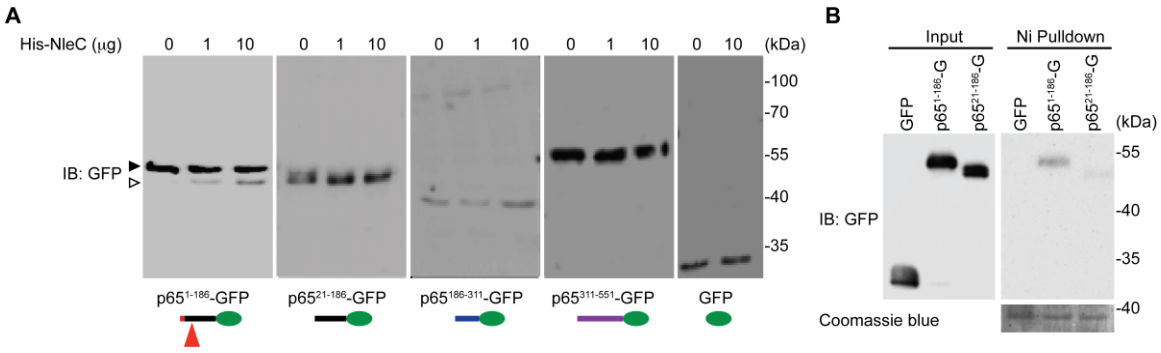


Figure 2.8 The location of the GFP tag does not alter NleC-mediated cleavage of $p65^{1-186}$

Various truncations of N- or C-terminally GFP-tagged p65, as indicated, were transfected into HEK293T cells. Whole cell lysates were subjected to the His-NleC cleavage assays, and immunoblotted with anti-GFP antibody for NleC-cleaved fragments. The cleaved fragments from GFP-p65 and $p65^{1-186}$-GFP proteins were labeled by filled and open triangles, respectively.

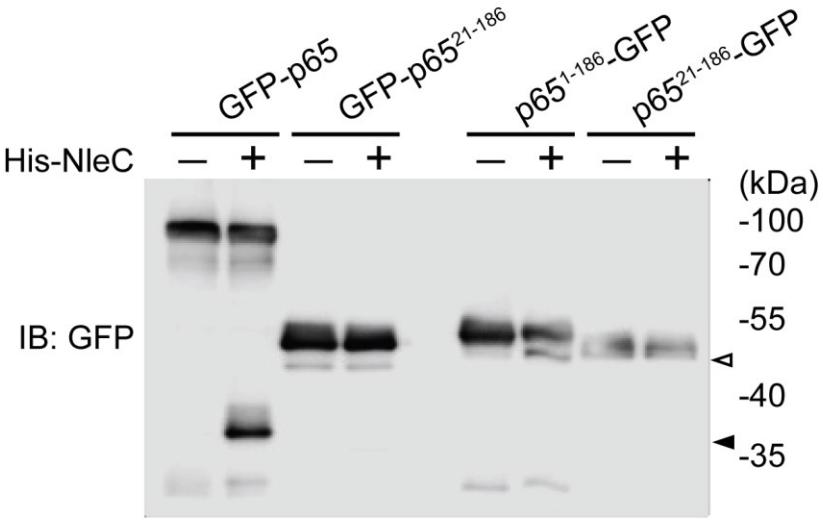


Figure 2.9 The N-terminal 20 amino acids are required for NleC to bind to p65

Whole cell lysates derived from HEK293T cells expressing the indicated GFP-tagged p65 proteins were incubated with His-NleC at 4°C. Nickel beads were added to pull-down His-NleC and associated proteins. Samples were separated by SDS/PAGE, followed by immunoblot for indicated proteins.

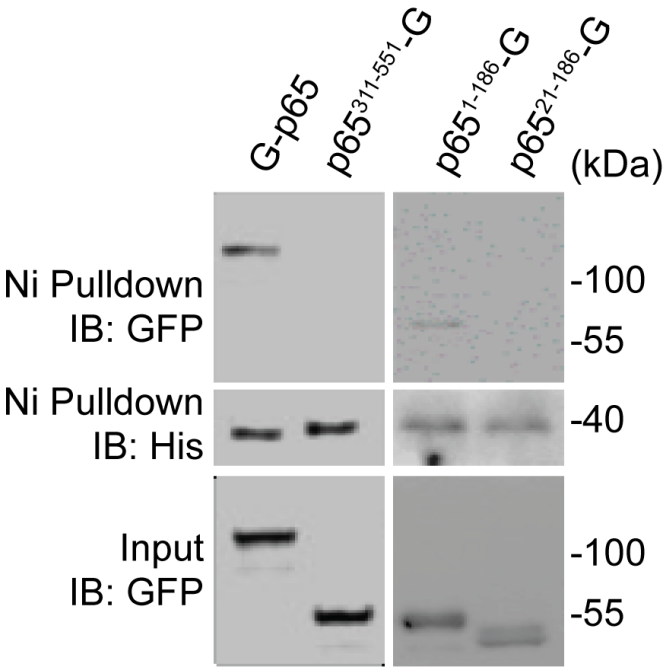


Figure 2.10 The dominant NleC cleave site on p65 is Cys38/Glu39.

A-B. Whole cell lysates derived from HEK293T cells expressing N-terminal GFP-tagged p65 were subjected to the cleavage assay using wild-type His-NleC or the catalytic mutant His-NleC (H117Y), and immunoblotted with p65 N-terminus specific antibody for NleC-cleaved p65 fragments. C. Schematic of proposed NleC cleavage sites on p65. D. The structure of N-terminus of p65, with dimerization domain (DimD), N-terminal domain (NTD), Cys38, and Glu39 highlighted in yellow, orange, red, and blue, respectively. Image was created from PDB file 1VKX (Chen et al., 1998) using the Pymol software. E. Whole cell lysates derived from HEK293T cells expressing wild-type or mutant GFP-tagged p65 as indicated were subjected to the NleC cleavage assay and immunoblotted with p65 N-terminus specific antibody for NleC-cleaved p65 fragments. The percentage of GFP-tagged full-length p65, $p65^{1-38}$ fragment, and $p65^{1-10}$ fragment among the total GFP-tagged p65 proteins under each condition was quantified by densitometry. F-G. Whole cell lysates derived from HEK293T cells were subjected to the His-NleC cleavage assays, and immunoblotted with p65 N-terminus (F) and C-terminus (G) specific antibodies for NleC-cleaved p65 fragments.

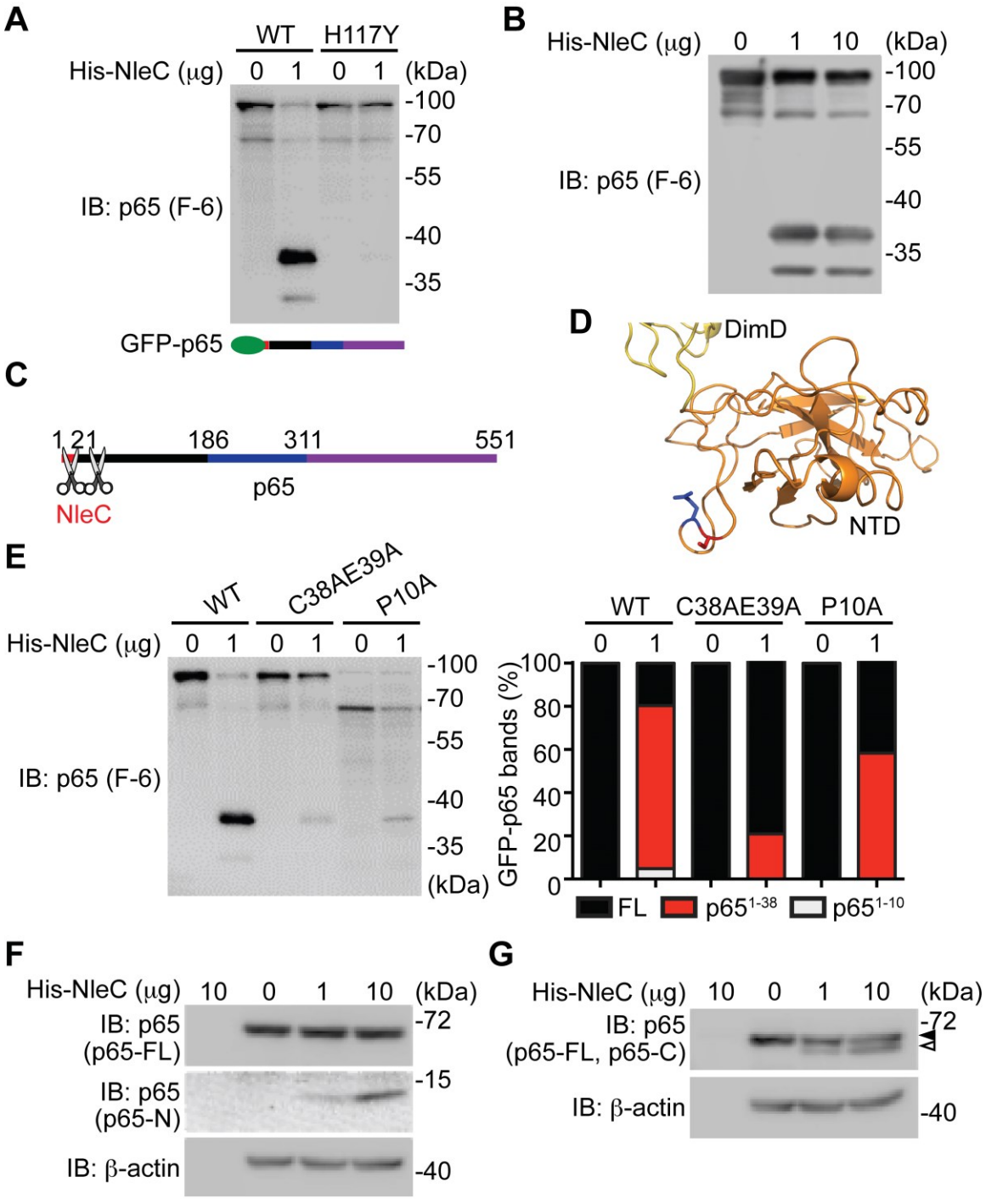

A
WT
H117Y
His-NleC (μg)
0 1 0 1 (kDa)
-100
-70
-55
IB: p65 (F-6)
-40
-35
GFP-p65
B
His-NleC (μg) 0 1 10 (kDa)
-100
-70
-55
IB: p65 (F-6)
-40
-35
C
1 21 186 311 551
p65
NleC
D
DimD
NTD
E
WT
C38AE39A
P10A
His-NleC (μg)
0 1 0 1 0 1
-100
-70
-55
IB: p65 (F-6)
-40
-35
(kDa)
WT C38AE39A P10A
0 1 0 1 0 1
GFP-p65 bands (%)
100
80
60
40
20
0
FL
p65 1-38
p65 1-10
F
His-NleC (μg) 10 0 1 10 (kDa)
IB: p65 (p65-FL)
-72
IB: p65 (p65-N)
-15
IB: β-actin
-40
G
His-NleC (μg) 10 0 1 10 (kDa)
IB: p65 (p65-FL, p65-C)
-72
IB: β-actin
-40

Figure 2.11 NleC cleaves p65 during *C. rodentium* and EPEC infections.

A. HEK293T cells expressing N-terminal GFP-tagged p65 were mock infected or infected with indicated *C. rodentium* strains at 100 MOI for 3 h. Whole cell lysates were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins. The GFP-tagged $p65^{1-10}$ cleavage products are indicated by an arrow, and an asterisk labels nonspecific bands. **B.** Caco-2 cells were mock infected or infected with indicated EPEC strains at 100 MOI for 1 h. Whole cell lysates were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins. **C.** Mouse colon epithelial cells (CECs) were mock infected or infected in suspension with indicated *C. rodentium* strains at 100 MOI for 3 h. Whole cell lysates were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins. The full-length p65 and cleaved p65 C-terminal fragment are indicated by filled and open triangles, respectively. **D.** Mouse CECs infected as in C were separated from free *C. rodentium* by Percoll gradient centrifugation. Whole cell lysates from CECs and attached *C. rodentium* were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins. **E.** Representative immunofluorescence micrographs of mouse CECs infected as in C that were centrifuged onto cover slips and stained for *C. rodentium* LPS, with nuclei counterstained by DAPI. Scale bars, 10 μm. **F.** The numbers of *C. rodentium* attached to mouse CECs as in E (from 6 random fields) were quantified and normalized to the perimeter of individual CEC.

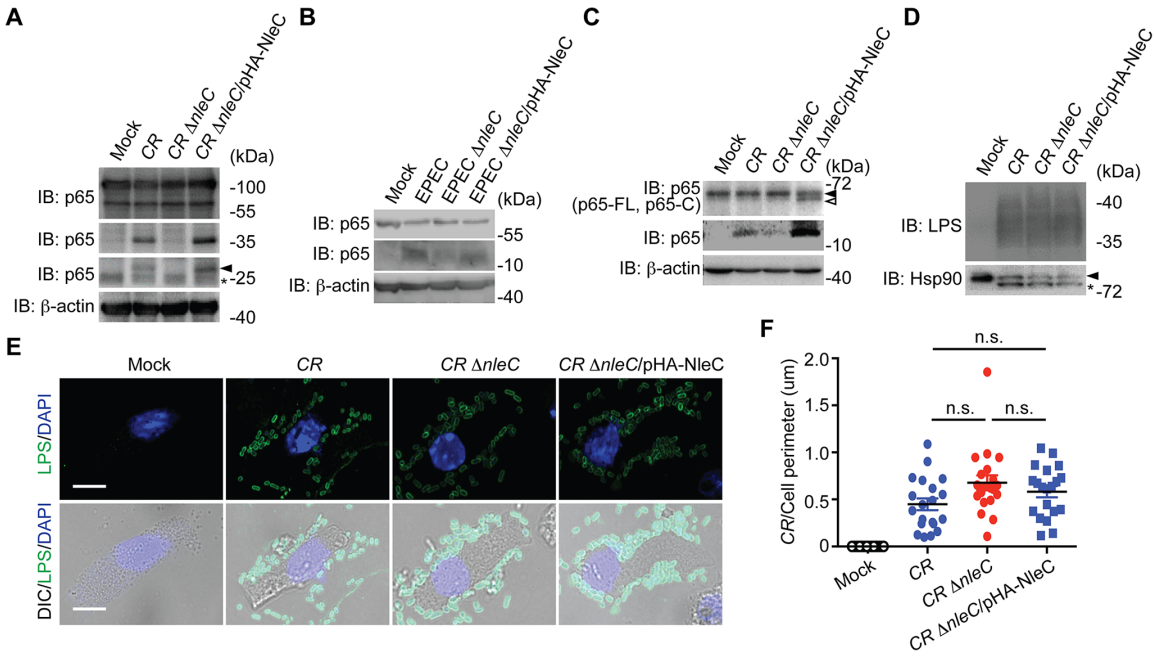

A
Mock
CR
CR ΔnleC
CR ΔnleC/pHA-NleC
(kDa)
IB: p65
-100
-55
IB: p65
-35
IB: p65
-25
IB: β-actin
-40
B
Mock
EPEC
EPEC ΔnleC
EPEC ΔnleC/pHA-NleC
(kDa)
IB: p65
-55
IB: p65
-10
IB: β-actin
-40
C
Mock
CR
CR ΔnleC
CR ΔnleC/pHA-NleC
(kDa)
IB: p65
(p65-FL, p65-C)
-72
IB: p65
-10
IB: β-actin
-40
D
Mock
CR
CR ΔnleC
CR ΔnleC/pHA-NleC
(kDa)
IB: LPS
-40
-35
IB: Hsp90
-72
E
Mock
CR
CR ΔnleC
CR ΔnleC/pHA-NleC
LPS/DAPI
DIC/LPS/DAPI
F
n.s.
n.s.
n.s.
CR/Cell perimeter (um)
2.0
1.5
1.0
0.5
0
Mock
CR
CR ΔnleC
CR ΔnleC/pHA-NleC

Figure 2.12 p50 is not cleaved during infection with EPEC or *C. rodentium*

A. Caco-2 cells were mock infected or infected with the indicated EPEC strains at 100 MOI for 1 h. Whole cell lysates were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins. **B.** Mouse colon epithelial cells (CECs) were mock infected or infected in suspension with the indicated *C. rodentium* strains at 100 MOI for 3 h. Whole cell lysates were derived, separated by SDS/PAGE, and immunoblotted for indicated proteins.

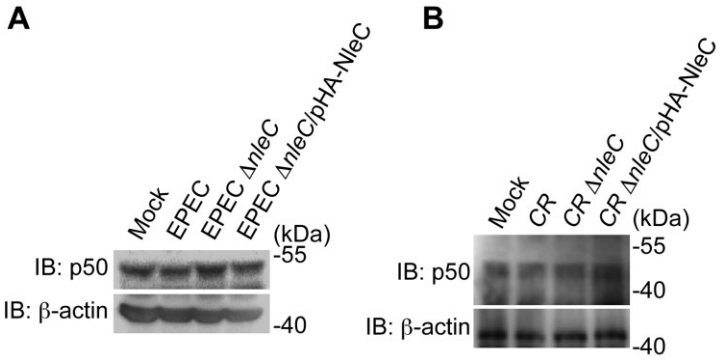


Figure 2.13 NleC suppresses inflammatory responses in *C. rodentium*-infected mice.

A. Representative immunofluorescence micrographs of *C. rodentium* LPS, with nuclei counterstained by DAPI, in colon sections derived from C57BL/6 mice 8 days post inoculation with the indicated strains of *C. rodentium*. Scale bars, 10 μm. Lu indicates the colon luminal space. **B.** Quantitative PCR (qPCR) was used to determine mRNA levels of indicated cytokine/chemokine genes relative to *Actb* (β-actin) in the colons collected from C57BL/6 mice 14 days post inoculation with wild-type or *ΔnleC* mutant strain of *C. rodentium*. **C-D.** Immunofluorescence micrographs of $CD11b^+$ inflammatory immune cells in the colons collected from C57BL/6 mice as in B, with nuclei counterstained by DAPI. Scale bars, 10 μm. The colon-infiltrated inflammatory immune cells (from 5 random fields) with CD11b staining were quantified (D). **E.** Hematoxylin and eosin (H&E) staining of colons collected from C57BL/6 mice as in D. Scale bars, 200 μm. **F.** The histopathology scores of colon sections derived from mice infected as indicated as in D. Shown are mean ± s.e.m of 10 random fields from two independent experiments.

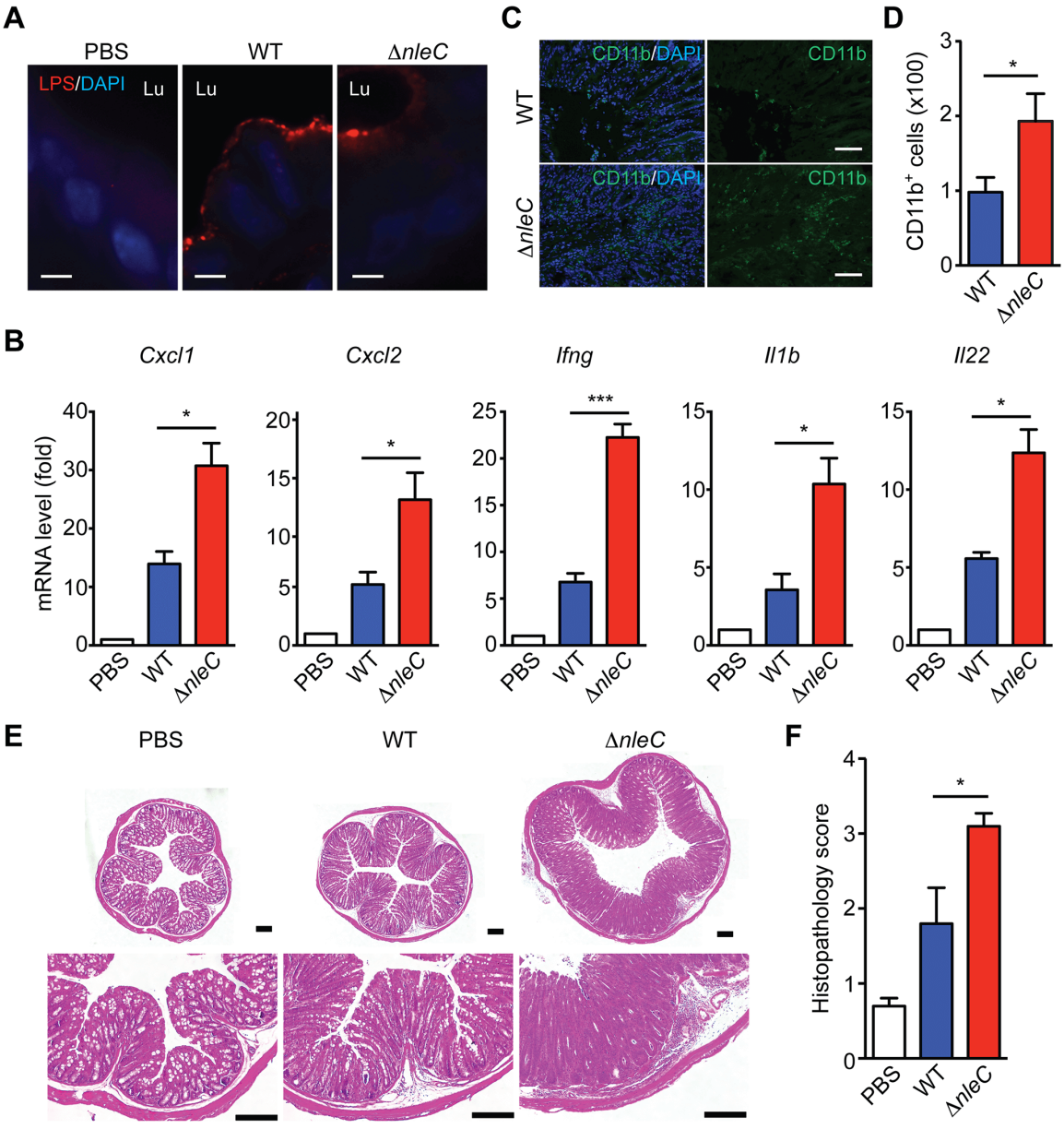

A
PBS
WT
ΔnleC
LPS/DAPI
Lu
Lu
Lu
C
CD11b/DAPI
CD11b
WT
ΔnleC
D
CD11b+ cells (x100)
WT
ΔnleC
B
Cxcl1
Cxcl2
Ifng
Il1b
Il22
mRNA level (fold)
PBS
WT
ΔnleC
E
PBS
WT
ΔnleC
F
Histopathology score
PBS
WT
ΔnleC

Figure 2.14 Wild-type and ΔnleC *C. rodentium* colonize mice equivalently

Colons were harvested from C57BL/6 mice at day 10-post inoculation with the indicated *C. rodentium* strains and cleaned of their contents, and homogenized in PBS. Serial dilutions were performed and plated on MacConkey agar plates. Colonies were counted to determine the CFU/g of colon tissue.

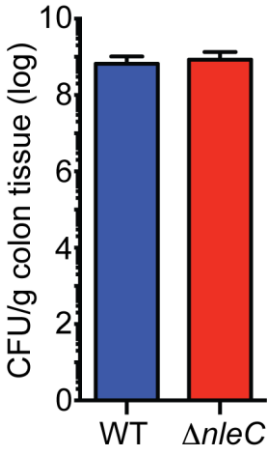


Figure 2.15 The $p65^{1-38}$ fragment interferes with the RPS3-p65 interaction in the cytoplasm and selectively attenuates the nuclear translocation of RPS3.

A. HEK293T cells were transfected with C-terminal GFP-tagged $p65^{1-38}$, $p65^{39-551}$, or GFP control plasmids. 28 h later, the cytosolic and nuclear fractions were derived and immunoblotted (IB) for indicated proteins. Hsp90 and PARP1 served as loading controls and cytosolic and nuclear markers, respectively. B. HEK293T cells were transfected with indicated GFP-tagged p65 or GFP control plasmids. 28 h later, whole cell lysates (Input) were derived and directly IB, or after immunoprecipitation (IP) with RPS3 antibody, for indicated proteins. Non-specific bands and immunoprecipitated GFP-p65 proteins are indicated by asterisks and arrows, respectively. HC, heavy chain; LC, light chain. C. HEK293T cells expressing GFP-tagged $p65^{1-38}$ or GFP alone were stimulated with 50 ng ml^{-1} of TNF for indicated periods. Whole cell lysates were derived and IB for IκBα, with β-actin as a loading control. D. HEK293T cells were transfected and stimulated as in C, and nuclear fractions were derived and IB for indicated proteins. Hsp90 and PARP1 served as loading controls and cytosolic and nuclear markers, respectively. E. HEK293T cells were transfected with increasing amounts of GFP-$p65^{1-38}$, compensated with GFP control, together with 5 × κB-Luc reporter and pTKRL plasmids. After 28 h, the cells were stimulated in the presence or absence of TNF (50 ng ml^{-1}) and analyzed for luciferase activity.

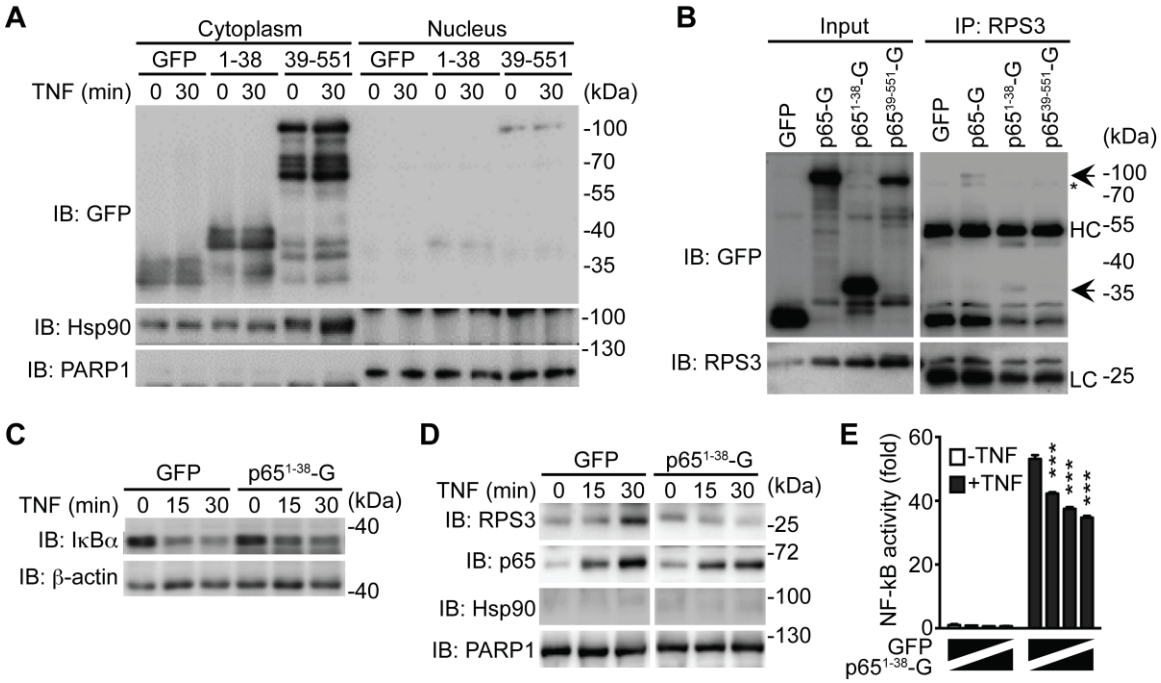

A
Cytoplasm
Nucleus
GFP
1-38
39-551
GFP
1-38
39-551
TNF (min)
0 30 0 30 0 30 0 30 0 30 0 30
(kDa)
IB: GFP
-100
-70
-55
-40
-35
IB: Hsp90
-100
IB: PARP1
-130
B
Input
IP: RPS3
GFP
p65-G
p65 1-38 -G
p65 39-551 -G
GFP
p65-G
p65 1-38 -G
p65 39-551 -G
(kDa)
IB: GFP
-100
-70
HC -55
-40
-35
IB: RPS3
LC -25
C
GFP
p65 1-38 -G
TNF (min)
0 15 30 0 15 30 (kDa)
IB: IκBα
-40
IB: β-actin
-40
D
GFP
p65 1-38 -G
TNF (min)
0 15 30 0 15 30 (kDa)
IB: RPS3
-25
IB: p65
-72
IB: Hsp90
-100
IB: PARP1
-130
E
NF-kB activity (fold)
60
40
20
0
-TNF
+TNF
***
***
***
GFP
p65 1-38 -G

Figure 2.16 The p65$^{1-38}$ binds to RPS3

Pull-down with recombinant GST or GST-RPS3 proteins with whole cell lysates derived from HEK293T cells expressing GFP-p65$^{1-38}$, followed by Ponceau S staining and immunoblotted (IB) for GFP-p65$^{1-38}$.

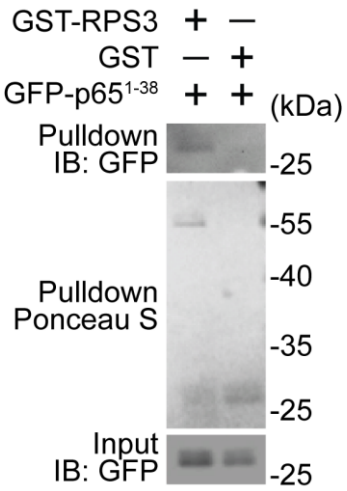
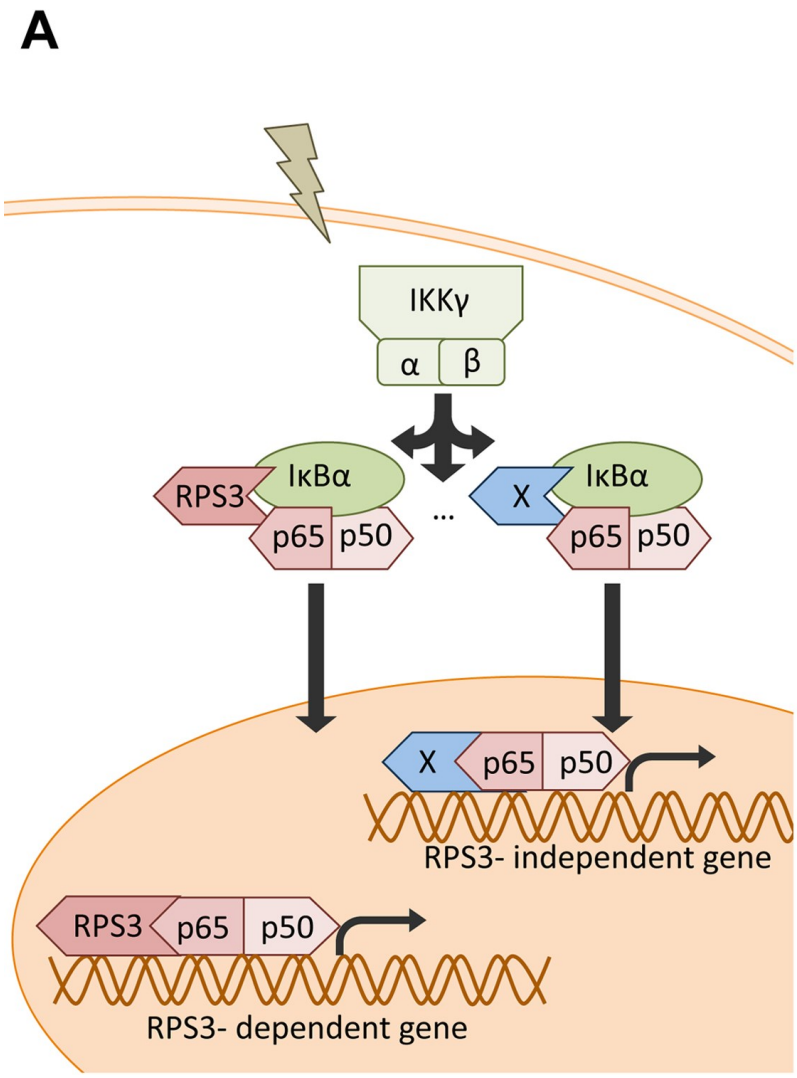
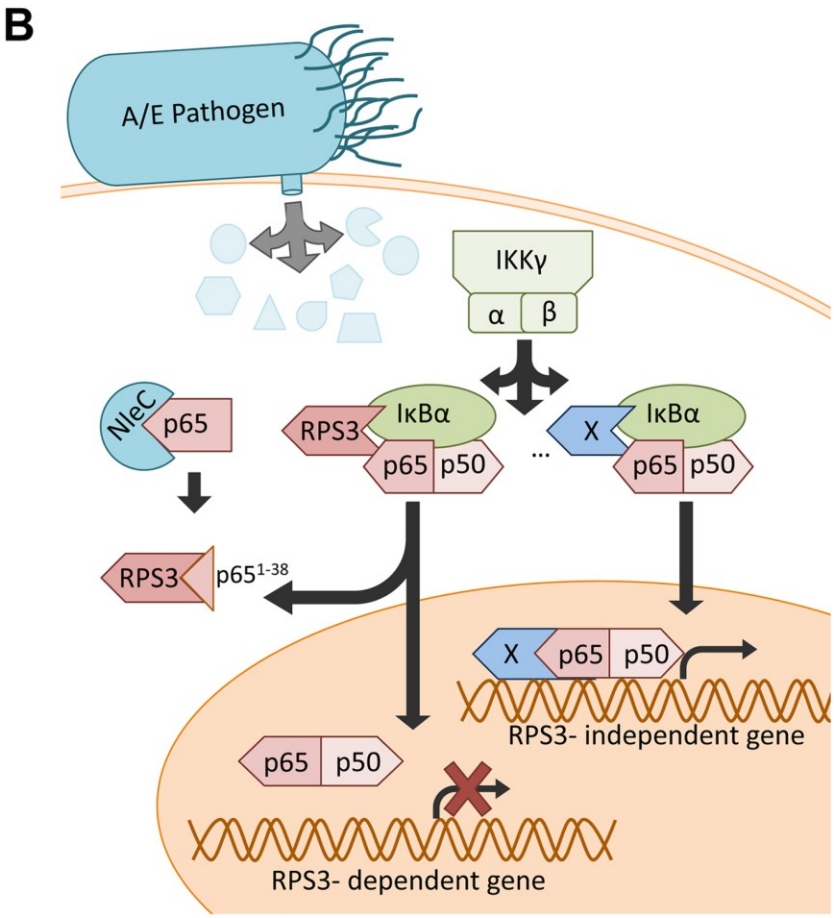


Figure 2.17 Schematic model of selective inhibition of NF-κB gene expression by the NleC-cleaved p65$^{1-38}$ fragment.

Under normal conditions (A), NF-κB stimuli activate both RPS3-dependent and -independent NF-κB signaling pathways. When injected into host cells via the T3SS during A/E pathogen infections (B), NleC cleaves a small percentage of p65, generating the p651-38 fragment that interferes with the RPS3-p65 interaction and sequestrates RPS3 in the cytoplasm. This leads to selective inhibition of the RPS3-dependent NF-κB gene transcription, without affecting the RPS3-independent gene transactivation.




B
A/E Pathogen
IKKγ
α
β
NleC
p65
RPS3
IκBα
p65
p50
...
X
IκBα
p65
p50
RPS3
p65^1-38
X
p65
p50
RPS3- independent gene
p65
p50
RPS3- dependent gene

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3 Interference with NF-κB signaling pathway by pathogen proteases: global and selective inhibition

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3.1 Summary

Pathogens have evolved a myriad of ways to abrogate and manipulate the host response to infections. Of the various mechanisms involved, pathogen-encoded and sometimes host-encoded proteases are an important category of virulence factors that cause robust changes on the host response by targeting key proteins along signaling cascades. The nuclear factor kappaB (NF-κB) signaling pathway is a crucial regulatory mechanism for the cell, controlling the expression of survival, immune, and proliferation genes. Proteases from pathogens of almost all types have been demonstrated to target and cleave members of the NF-κB signaling pathway at nearly every level. This review provides discussion of proteases targeting the most abundant NF-κB subunit, p65, and the impact of protease-mediated p65 cleavage on the immune responses and survival of the infected host cell. After examining various examples of protease interference, it becomes evident that the cleavage fragments produced by pathogen-driven proteolytic processing should be further characterized to determine whether they have novel and unique functions within the cell. The selective targeting of p65 and its effect on gene transcription reveals unique mechanisms by which pathogens acutely alter their microenvironment and further research may open new opportunities for novel therapeutics to combat pathogens.

3.2 Introduction

Epithelial cells and innate immune cells stand as the first line of defense against tissue damage and invading pathogens at mucosal surfaces. Encoding a variety of pattern recognition receptors (PRRs) that are capable of responding to an even greater variety of pathogen-associated molecular patterns (PAMPs), mucosal epithelial cells initiate immune responses. Stimulation of PRRs results in the activation of signaling cascades, through adaptor proteins, that transduce the signal to the transcription factor level. Most commonly, key transcription factors, including nuclear factor kappaB (NF-κB), activator protein 1 (AP-1), cAMP response element-binding protein (CREB), and interferon-regulatory factor (IRF), are the targets of these signaling pathways, resulting in the

transactivation of an array of target genes. The activation of inflammatory and anti-apoptotic proteins ultimately alerts the immune system of invasion and induces the recruitment of leukocytes to the site of infection. Cell surface receptors, such as toll-like receptors (TLRs), stimulate the cellular signaling and activation of NF-κB when the corresponding ligand binds its receptor. The NF-κB signaling pathways are crucial for the host response, as they serve as a master regulator and orchestrator of both the innate and adaptive immune responses (Finlay & McFadden, 2006, Le Negrate, 2012), making it a key target for pathogen interference (Thaker, 2014, Vallabhapurapu & Karin, 2009). A great number of previous studies have elucidated the mechanism by which the NF-κB pathways are regulated in normal and disease conditions (Hayden & Ghosh, 2012, Lenardo & Baltimore, 1989). Typically, NF-κB activation is initiated by ligand binding to a membrane bound cytokine or PRR receptor whose signal is transduced by the adaptor protein myeloid differentiation primary response gene 88 (MyD88) and others until the IκB kinase (IKK) complex is activated. The IKK beta subunit (IKKβ) phosphorylates and marks IκB for proteasomal degradation, which in turn unmasks the nuclear localization signal (NLS) of NF-κB thus liberating the NF-κB complex for translocation into the nucleus. Once in the nucleus, the NF-κB complex binds to a variety of specific double-stranded DNA sequences, termed κB sites, in the genome and recruits the general transcriptional machinery to induce the transcription of target genes (Lenardo & Baltimore, 1989), including those controlling immune responses and survival of host cells.

Besides the best-characterized Rel family proteins, i.e. p65 (also named RelA), RelB, c-Rel, p50, and p52, other non-Rel protein subunits in NF-κB complexes play an important role in optimizing the binding of target DNA and transcription of target genes. Ribosomal protein S3 (RPS3) was identified as the first 'specifier' subunit of NF-κB that confers the promoter selectivity and transcriptional specificity of NF-κB. RPS3 occurs along with p65 and IκBα in a portion, but not all, of the inhibitory NF-κB complexes in the cytoplasm (Wan et al. unpublished results). Once activated, RPS3 translocates, independent of p65, into the nucleus where it increases the affinity of

p65/p50 to particular κB sites (Wan et al., 2007, Wan et al., 2011). RPS3 functions as a 'specifier' of NF-κB gene transcription, shedding light on a major lingering question within the field regarding promoter selectivity and transcriptional specificity, especially considering how diverse stimuli generate specific responses amongst the large number of κB binding sites scattered throughout the genome. Moreover, this additional binding partner strengthens the association of the p65/p50 dimer at the particular κB sites of a growing list of genes that are now considered RPS3-dependent genes, allowing the activation of only a subset of the vast potential p65-dependent genes (Wan et al., 2007, Wan et al., 2011, Sen et al., 2012). Since then, other specifiers have been attributed to helping hone the activation of NF-κB-dependent genes. Src-associated substrate during mitosis of 68 kDa (Sam68) has been discovered as another non-Rel subunit of p65, functioning in the nucleus to mediate the binding of NF-κB to the CD25 promoter in activated T cells (Fu et al., 2013), and Kruppel-like factor 6 (KLF6) has been identified as another nuclear co-activator of NF-κB for a subset of genes (Zhang et al., 2014). The discovery of these specifier proteins not only serves to answer the question of promoter selectivity but also emphasize that while NF-κB dimer binding is required at the promoter, it is not sufficient to induce the transcription of particular genes. The specifier proteins function to promote the binding and enhance the efficiency of transcriptional activation of particular genes over others. The function of the currently known specifier proteins in particular inflammatory settings or specific tissue/cell types remains unknown. The existence of cell type specific responses to similar stimuli is well recognized and exemplified by the discussion of how gut epithelial cells tend to response to infection with a mainly anti-apoptotic profile (and some inflammatory responses), whereas the infiltrating immune cells build the majority of the inflammatory response (Spehlmann & Eckmann, 2009, Eckmann & Neish, 2011). Understanding the cell type specific responses to stimuli is particularly important when many pathogens have evolved preferences for infecting particular cell types which presumably aides their survival within the host.

As a result of the many host protective mechanisms, pathogens have co-evolved many strategies to overcome these host defense mechanisms. Encoding virulence factors, such as toxins, adhesins, secretion systems, decoy receptors, kinases, proteases, and compounds, that modulate host immune responses have resulted in the ability to greatly manipulate the host response (Casadevall & Pirofski, 2009). Pathogens express an array of virulence factors that change as the pathogen progresses through its life cycle/infection cycle. These factors target all aspects of the cellular immune responses to infection, however those that target proteins where intracellular signaling cascades converge are arguably the most challenging for host cells to overcome. This is likely because many surface receptors rely on a smaller pool of adaptor proteins, that if disrupted, prevent the relay of signals from the outside of the cell to the nucleus (Silke & Hartland, 2013), therefore the pathogen does not need to encode a virulence factor to target all cellular receptors, when it can target a common adaptor protein to turn off the cellular signaling cascades. Whether the pathogen is intracellular or extracellular and/or encodes a secretion system capable of translocating proteins into a host cell, it can gain access to the major signaling junctions employed by the mammalian host to orchestrate immune responses (Brodsky & Medzhitov, 2009). Due to the convergence of many PRR signaling cascades on proteins such as IKK and mitogen activated protein kinase (MAPK), it is more effective for pathogens to target the intracellular activator than attempt to block all the extracellular receptors (Silke & Hartland, 2013). With this strategy, pathogens can exact robust effects on cell signaling while encoding fewer effectors. Along with targeting a myriad of signaling pathways, pathogens also encode virulence factors with redundant functions in an effort to abrogate the host-signaling cascade. Many pathogens have acquired sophisticated mechanisms to directly interfere with the host NF-κB signaling pathways through regulating or mimicking host proteins to their own advantage, highlighting the importance of NF-κB signaling. Bacteria, viruses, and parasites encode virulence factors that operate at multiple levels of the sequential process of NF-κB signaling using redundancy to ensure blockade of the pathway, demonstrating how crucial inhibiting this particular pathway is for pathogen survival. Of note, deubiquitinating enzymes (Ndubaku & Tsui, 2015),

kinases (Krachler et al., 2011), proteases, and other encoded proteins are key virulence factors employed by pathogens to modulate NF-κB signaling in host cells. Of these, proteases are of particular interest because they alter the inherent structure of host proteins, thus preventing their native functions, although the cleaved fragments may play additional roles within the cell. This review will focus on the targeting of the NF-κB signaling pathway by intracellular proteases encoded by various pathogens. The regulation of NF-κB and the activation of downstream gene targets remain of great interest in host-pathogen interactions, but understanding how pathogens are manipulating these interactions to abrogate intracellular NF-κB signaling and dampen immune responses is paramount.

3.3 Targeting the upstream signaling molecules in the NF-κB pathway

Upon receptor engagement, the activation of adaptor proteins occurs to start the NF-κB signal transduction cascade in the cytoplasm that leads to changes at the transcriptional level of specific NF-κB target genes in the nucleus. The NF-κB signaling cascade contains many intermediate molecules (as well as important post-translational modifications) that are subject to pathogen intervention, in particular pathogen-encoded protease virulence factors. The Toll–interleukin 1 receptor (TIR) domain-containing proteins TRIF and MyD88, both of which are important adaptor proteins residing just inside the cellular membrane, were reported to be targeted by the NS3/4A protease encoded by Hepatitis C virus (HCV) (Li *et al.*, 2005a) and the 3C protease encoded by enterovirus 68 (Xiang *et al.*, 2014), whereas infection by the bacterium *Yersinia enterocolitica* activates human Caspase 3 to cleave these host proteins (Novikova *et al.*, 2014) . Targeting TRIF prevents signal transduction from TLR3 and TLR4, whereas abrogating MyD88 blocks signal transduction from most surface stimulation, as it is employed by all TLRs (except TLR3) and some cytokine and chemokine receptors (Kawai & Akira, 2010, Piras & Selvarajoo, 2014). Thus the protease-mediated cleavage of TRIF and MyD88 has an extensive effect on downstream pathways, as evidenced by the lack of NF-κB activation during HCV (Li et al., 2005a), enterovirus 68 (Xiang et al., 2014), and *Y.*

enterocolitica (Novikova et al., 2014) infections. Of note, besides the NF-κB signaling pathway, the NS3/4A and 3C proteases also block the activation of the interferon response by targeting TRIF (Kawai & Akira, 2010, Piras & Selvarajoo, 2014). As a result, these pathogens have robust effects on host defense with minimal energy input, by encoding a single protease that can target the nexus of multiple signaling pathways. Moreover, HCV is especially adept in this respect, as the same protease also targets the intracellular adaptor mitochondrial antiviral-signaling protein (MAVS) (Li *et al.*, 2005b, Meylan *et al.*, 2005) that is crucial for RIG-I helicase-initiated activation of NF-κB and interferon responses (Yoneyama & Fujita, 2007). Moreover, the porcine reproductive and respiratory syndrome virus (PRRSV) encoded protease NSP4 has been shown to cleave NEMO/IKKγ (Huang *et al.*, 2014), a regulatory subunit of the IKK complex that serves as the master NF-κB regulator that is activated by the stimulation of various cell surface molecules. Furthermore, the 3C protease encoded by Coxsackievirus has been demonstrated to cleave IκBα (Saura *et al.*, 2007, Zaragoza *et al.*, 2006), which sequesters NF-κB in the cytoplasm. Interestingly, the cleavage of IκBα by Coxsackievirus 3C protease does not completely degrade IκBα, something that would lead to the full liberation and constitutive activation of NF-κB. Instead, the proteolytic fragment, remains bound to and translocates into the nucleus with NF-κB complexes, dominantly interfering with NF-κB DNA binding capability, thus dampening the NF-κB activation in host cells during Coxsackievirus infection (Saura et al., 2007, Zaragoza et al., 2006). These pathogen-encoded proteases and their proposed target proteins in host cells are summarized in **Table 1**. By targeting the upstream nodes of this signaling cascade, these proteases are capable of causing a global suppression of the NF-κB signaling pathway specifically, as well as other signaling cascades that crosstalk via some of these adaptor molecules.

3.4 p65, a major target of pathogen proteases

In addition to targeting various key molecules upstream of the NF-κB signaling pathway, pathogens have acquired strategies to interfere with NF-κB, in particular the Rel subunits that are

directly involved in binding to DNA promoters of various target genes. Although there is some cell type variability, the most abundant NF-κB species in a cell consists of p65, p50, and other proteins (Wan et al., 2007). The p65 protein also possesses a transactivation domain (TAD) that is crucial for recruiting the general transcriptional machinery for the transactivation of target genes (Wan & Lenardo, 2010). These features make p65 unique among Rel family proteins and most frequently modulated by many pathogens including bacteria, parasites, and viruses..

3.4.1 Bacteria encoded proteases

Pathogenic bacteria encoding a membrane-bound protein complex, termed the type III secretion system (T3SS), such as the attaching/effacing (A/E) pathogens including enteropathogenic *Escherichia coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), and the mouse pathogen *Citrobacter rodentium*, are prime examples of pathogens that interact with host intracellular signaling cascades to alter their microenvironment (Hartland & Leong, 2013, Shames & Finlay, 2012, Silke & Hartland, 2013, Wong *et al.*, 2011). EHEC, EPEC, and *C. rodentium* encode a long list of virulence proteins (effectors) that are delivered into host cells during infection via the T3SS. These effectors target multiple cellular pathways present within the cell and function to manipulate the host cellular signaling, often with multiple effectors targeting the same pathway (reviewed in (Wong et al., 2011)). As such, the non-LEE-encoded (Nle) effectors NleB, NleE, NleH1, and NleC all target the NF-κB signaling pathway, of which NleB blocks the signal transduction between the TNF receptor-associated factor 2 (TRAF2) and NF-κB upstream proteins by preventing the ubiquitination of TRAF2 (Pham *et al.*, 2013); NleE modifies TGF-β Activated Kinase 1 Binding Protein 2/3 (TAB2/3) that transduces the activating signal from a cytokine receptor to TGF-β Activated Kinase 1 (TAK1) or IKK kinases (Yao *et al.*, 2014); NleH1 inhibits the phosphorylation of RPS3 thereby preventing its nuclear translocation and 'specifier' activity (Gao *et al.*, 2009). Using multiple effectors to block NF-κB signaling allows the pathogen to regulate its microenvironment and ensure particular host signal transduction pathways are blocked. Another effector targeting NF-κB is the metalloprotease NleC,

which was shown to cleave mainly p65 (Baruch *et al.*, 2011, Hodgson *et al.*, 2015, Li *et al.*, 2014, Muhlen *et al.*, 2011, Pearson *et al.*, 2011, Turco & Sousa, 2014, Yen *et al.*, 2010), although p300 was also proposed (Shames *et al.*, 2011), in host cells. As a canonical zinc metalloprotease with the conserved HEXXH motif, NleC is well conserved among EHEC, EPEC, and *C. rodentium (Turco & Sousa, 2014)*. NleC was first described as an important effector for the infection-mediated suppression of interleukin-8 (IL-8) in host cells (Yen et al., 2010, Sham *et al.*, 2011) and subsequently revealed by multiple groups to cleave p65 in order to suppress host inflammatory responses (Baruch et al., 2011, Hodgson et al., 2015, Li et al., 2014, Muhlen et al., 2011, Pearson et al., 2011, Turco & Sousa, 2014, Yen et al., 2010). Moreover, the molecular details about the interaction between p65 and NleC have also been illustrated by recent crystal structural analyses showing that the complimentary electrostatic interactions surrounding the catalytic zinc binding site of NleC and the DNA binding loop of p65 (Li et al., 2014) and that NleC structurally and chemically mimics DNA to exploit the DNA binding motifs of p65 (Turco & Sousa, 2014). Although different cell lines and pathogens were utilized, the cleavage sites of p65 by NleC from previous studies appear to occur between proline 10 and alanine 11 (P10/A11) (Yen et al., 2010), cysteine 38 and glutamic acid 39 (C38/E39) (Baruch et al., 2011, Li et al., 2014, Turco & Sousa, 2014), or both with C38/E39 being the primary site (Hodgson et al., 2015). More importantly, the C38/E39 cleavage site on p65 is of particular interest as C38 sulfahydration was recently proposed to be a crucial post-translational modification to stabilize the p65-RPS3 interaction, thus facilitating RPS3/p65-dependent NF-κB target gene transcription (Sen et al., 2012).

The gram-negative bacterium *Photobacterium damselae piscicida* (*Phdp*) that causes fish pasteurellosis, a disease characterized by septicemia and high mortality is a considerable threat for mariculture (Barnes *et al.*, 2005, Romalde, 2002). *Phdp* encodes an A-B toxin termed AIP56, whose N-terminus is homologous to NleC, maintaining the conserved HEIVH zinc binding domain, whereas the C-terminus serves as an internalization mechanism for the secreted AIP56 to enter target cells (Silva *et al.*, 2013), macrophages and neutrophils (do Vale *et al.*, 2007, do Vale *et al.*, 2003).

During *Phdp* infection, macrophages and neutrophils, the primary cell type employed by the immune system to clear apoptotic cells before secondary necrosis, undergo AIP56-dependent apoptosis (do Vale et al., 2007, Silva et al., 2013), causing the release of their cytotoxic components thus resulting in an inflammatory response (do Vale et al., 2007, do Vale et al., 2003). Given the homology between NleC and AIP56, it is not surprising that AIP56 also targets the same Cys/Glu residues, *i.e.* C39/E40 in fish, within the Rel homology domain (RHD) of p65 for cleavage. The apoptotic effect executed by AIP56 is thought to result from the abrogated gene transcription of anti-apoptotic genes normally conferred by NF-κB/p65 (Silva et al., 2013). In this setting, blocking the NF-κB-mediated anti-apoptotic gene transcription induces an overwhelming immune response and severe immunopathology in fish hosts subsequent to *Phdp* infection.

The well known intracellular bacteria, *Chlamydia trachomatis,* is the primary cause of preventable blindness worldwide as well as the most commonly found sexually-transmitted disease in the United States (Brunham & Rey-Ladino, 2005), which makes it a pathogen of intense clinical interest. Although the murine infection model nicely recapitulates the acute genital tract infection observed in women, spontaneous clearance and life long immunity make the mouse disease differ from the human disease in which infections can last several months and re-infection is common (Morrison & Caldwell, 2002). As an intracellular pathogen, *C. trachomatis* employs several proteases at various life stages to regulate host-cellular signaling. CT441 is a tail-specific protease (Tsp) containing a PDZ (postsynaptic density protein [PSD95], Drosophila disc large tumor suppressor [Dlg1], and zonula occludens-1 protein [zo-1]) domain and conserved serine and lysine residues that serve as the catalytic unit. CT441 shares approximately 70% sequence identity around the catalytic unit with other PDZ domain-containing Tsp proteases (Lad *et al.*, 2007b). Lad and colleagues reported that CT441 cleaves human p65, however the murine protein is resistant to cleavage, due to several differences in their amino acid sequences between residues 331-359 (Lad et al., 2007b). Moreover, swapping the residues 331-359 between human and murine p65 proteins renders the human p65 resistant to CT441 cleavage. Of note, selectively cleaving only the human protein versus the mouse protein could

serve as a key-determining factor explaining the disease difference between the two species. It has been speculated that without cleaving p65, the mouse immune response may be induced early enough to prevent widespread dissemination of the *Chlamydia* bacteria, leading to an accelerated clearance. Lad and colleagues also demonstrated that expression of CT441 not only induced cleavage of human p65 but also prevented NF-κB activation, as illustrated by a lack of IκBα degradation and p65 nuclear translocation (Lad *et al.*, 2007a). Cleavage of p65 could result in the lack of p65 nuclear translocation; moreover, the cleaved N-terminus of p65, defined as p40, was proposed to directly interact with IκBα, adding additional dominant negative regulation to NF-κB activation. The p40-IκBα interaction was hypothesized to protect IκBα from degradation, although the protective mechanism is unclear and there exists the possibility that another effector encoded by *C. trachomatis* has a role in protecting IκBα from degradation (Lad et al., 2007a). Indeed, targeting the same signaling pathway via multiple strategies is not uncommon for many pathogens, as such redundancy ensures full manipulation of the host microenvironment to the pathogens' advantage. It remains to be determined whether the lack of IκBα degradation and subsequent NF-κB activation are also restricted to human infection, but not in the murine infection. Further inquiry would further clarify the cause of the IκBα protection being dependent on the presence of the p40 cleavage product, since only the human p65 can be cleaved to generate the p40 product.

3.4.2 Parasite encoded proteases

The *Leishmania* parasite is responsible for the infection of more than 2 million people around the world every year (de Vries *et al.*, 2015). Spread by the *Phlebotomus* sandfly, this vector borne parasite has many life stages which vary in their expression and activation of various proteins, constantly providing a moving target for treatment and vaccine development (Jain & Jain, 2015). It was reported that the *Leishmania* metalloprotease gp63 cleaves p65 and produces a stable truncated form of p65, termed $p35^{RelA}$, which executes a functional role within the cell (Gregory *et al.*, 2008a). The p35 fragment retains the nuclear translocation ability of the full-length protein where it

heterodimerizes with p50 and binds DNA. Surprisingly, despite both proteins lacking a TAD domain, the p35 truncation and p50 heterodimer are still capable of turning on the expression of a particular set of chemokines (Gregory et al., 2008a). This indicates that beyond NF-κB, additional transcriptional factor(s) that possess a TAD may be involved in the transcription of these chemokine genes, considering that it is known that NF-κB couples with other transcription factors such as AP-1 and STATs to form a higher-order enhanceosome for optimizing the gene expression in immune responses (Fan *et al.*, 2010, Fujioka *et al.*, 2004, Neff *et al.*, 2001). Previous studies showed that infection with *Leishmania* parasites resulted in the failed activation of macrophages and proinflammatory signaling. Moreover, infected macrophages have also been revealed to be defective in reactive oxygen species (ROS) production and chemotaxis (Gregory *et al.*, 2008b, Olivier *et al.*, 2005). However, it remains unknown how the expression of certain chemokines, such as macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and Regulated on Activation, Normal T Cell Expressed and Secreted (RANTES) are induced during *Leishmania* infection (Dasgupta *et al.*, 2003, Olivier et al., 2005). In particular the transactivation of these chemokines is a survival strategy for the parasite, as the recruitment of more macrophages to the infection site allows the parasite to move into a new host cell and continue the infection cycle (Dasgupta et al., 2003). Gregory and colleagues demonstrated that the cleaved p35 fragment induced chemokine gene transcription, however, little more mechanistic detail beyond DNA binding was offered. The authors speculated that the remaining molecules of the p35 fragment present within the nucleus could be acting in a dominant negative capacity to block the transactivation of other NF-κB target genes, such as *Nos2*, encoding inducible nitric oxide synthase that would be detrimental to the parasite (Gregory et al., 2008a). In this way, the p35 fragment may be acting in a repressive manner, similar to how p50 homodimers are thought to act, as they also lack the TAD domain. Hence, gp63 induced cleavage of p65 provides the parasite with the ability to acutely modify its microenvironment by regulating NF-κB target gene transcription.

3.4.3 Viral encoded proteases

Viruses also encode various virulence factors that allow them to manipulate the crucial signaling pathways in host cells, which is more critical for viruses than other pathogens as viruses solely rely on host cells for replication. For instance, all members of the picornavirus family encode a 3C cysteine protease that processes the viral RNA encoded polyprotein (reviewed in (Palmenberg, 1990)). Among them, it has been demonstrated that poliovirus, as well as a few others, targets NF-κB p65 in order to shut down aspects of the innate immune response in host cells (Neznanov *et al.*, 2005). Poliovirus infects humans via the gastrointestinal tract and enters the bloodstream via the local lymphoid tissue. Most infections are asymptomatic or mild; however in a subset of individuals the virus eventually infects cells within the central nervous systems (CNS) where it causes the characteristic paralysis known as poliomyelitis. *In vitro,* poliovirus infection of host cells causes a short-lived activation of NF-κB, as demonstrated by nuclear translocation of p65/p50 and IκBα degradation peaking at about 4 hours post infection (Neznanov et al., 2005). Interestingly, around 3 hours post infection, the appearance of an N-terminal truncation of p65, termed p65ΔC, occurs simultaneously with the translation and detection of the poliovirus-encoded 3C protease (Neznanov et al., 2005). It was further demonstrated that the cleavage product is a result of the 3C protease and not the induction of apoptosis, which is also supported by the careful control over the timing and induction of apoptosis that poliovirus infection is able to exert on the cell (Agol *et al.*, 2000, Levkau *et al.*, 1999). Indeed, the control of apoptosis and the initial activation of the NF-κB signaling pathway, which causes the expression of some anti-apoptotic genes, allow the host cell to survive long enough for the virus to replicate its genome. While poliovirus is a well-studied example of the picornavirus family, Neznanov and colleagues also showed that other representatives of the family encode 3C, such as ECHO-1 and rhino-14, and can also cleave p65 (Neznanov et al., 2005). Elucidating the spatial-temporal regulation of these virulence factors is needed in future studies to allow for a deeper understanding of how these many strategies and regulatory pathways interact within host cells.

3.4.4 Host-encoded proteases benefiting pathogens

Besides interfering with host signaling pathways directly with pathogen-encoded proteases, pathogens take advantage of host protein functions (NF-κB being one of them) to serve their own needs. This is best exemplified by the human immunodeficiency virus (HIV). Indeed, the HIV genome encodes and requires NF-κB binding sites to boost the transcription of certain viral proteins (Alcami *et al.*, 1995, Bachu *et al.*, 2012, Coiras *et al.*, 2008, Perkins *et al.*, 1993, Zhang *et al.*, 2012). Since HIV primarily infects and replicates in activated T cells, Coiras and colleagues characterized the effects of T cell activation on the NF-κB signaling pathway (Coiras et al., 2008). Interestingly, they uncovered that immediately after the phorbol myristate acetate (PMA) or phytohemagglutinin (HA) stimulation of T cells, an apoptosis-unrelated, low-level activation of Caspase-3 leads to proteolytic processing of p65. The group further characterized the fate of the cleaved C-terminal product of p65, containing amino acids 97-551 and termed ΔNH_2p65. They demonstrated that ΔNH_2p65 bound to IκBα and caused constitutive activation of p65 by protecting the full-length p65 from IκBα inhibition, ultimately leading to an increase in HIV viral replication (Coiras et al., 2008). While the HIV-encoded proteins themselves are not causing the cleavage of p65, the virus's preference for a cell type that innately cleaves a low level of p65 upon activation, is indicative of the advantage HIV gains by replicating in such a host cell. The careful balance between activating Caspase-3 at low levels to cleave some of the p65 molecules within T cells and inducing extensive apoptosis should be highlighted here as too much Caspase-3 activity could certainly backfire on the virus. Anti-apoptotic genes would still be transactivated in such an infected cell, as most of the p65 molecules in the cell are left intact and constitutive NF-κB activation would induce their expression, which therefore results in the maintenance of an infected cell with enough host signaling left intact to produce more viral copies and propagate the infection.

3.5 NF-κB/p65 cleavage: linear or selective inhibition?

As supported by the previous cited studies, it is apparent that pathogen-encoded protease mediated cleavage of a variety of signaling molecules in host cells, especially the NF-κB signaling pathway (as summarized in **Table 2**), executes a dramatic and robust impact on the host immune responses. In most cases, it is proposed that the cleavage of a single key molecule in the NF-κB signaling pathway, along with other encoded virulence factors, is sufficient to block the signal transduction necessary to activate NF-κB target genes, especially proinflammatory cytokine/chemokine genes, thus modulating the global host immune responses to the pathogen's advantage and allowing the persistence of the infection (**Figure 1**). This explanation, at first glance, appears to be a reasonable incorporation of observations made during pathogen infection; however, it falls short in clarifying the dramatic impact on host inflammatory cytokine/chemokine gene expression when p65 is cleaved during the *bona fide* pathogen infections. A great number of studies have established that NF-κB is acutely and robustly activated during pathogen infections, as illustrated by a rapid nuclear translocation of the majority of p65, as well as the other Rel proteins, in host cells (Hayden & Ghosh, 2012, Li & Verma, 2002, Pott & Hornef, 2012, Sun & Andersson, 2002, Thaker, 2014). This is further emphasized by the need to encode a series of virulence factors that target multiple levels of the same signaling pathway as has been demonstrated by the A/E pathogens (Wong et al., 2011). If each p65 molecule in the host cell makes an equal contribution to the transactivation of NF-κB-targeted genes, pathogen-encoded proteases would have to cleave the majority, or even the entirety of the p65 pool in the cell to reach a substantial suppression of cytokine/chemokine gene transcription. However, most of the reported protease-mediated cleavage events in infected host cells have been characterized qualitatively, focusing on the identification of target host protein(s), but lacking further definition quantitatively. Even under conditions such as ectopic expression in host cells and *in vitro* protease assays with recombinant proteins, only a percentage of the entire p65 molecule pool in host cells is cleaved by the pathogen-encoded protease in question, leaving a substantial amount of intact full-length p65

(Baruch et al., 2011, Coiras et al., 2008, Hodgson et al., 2015, Li et al., 2014, Muhlen et al., 2011, Silva et al., 2013, Turco & Sousa, 2014, Yen et al., 2010). Unsurprisingly, the amount of p65 cleavage is even lower under the pathophysiological conditions of pathogen infection *in vivo* where the expression level of the protease is likely lower (Deng *et al.*, 2010). Hence, it remains puzzling how incomplete cleavage of p65 by many pathogen-encoded proteases has a disproportionally dramatic impact on NF-κB-mediated gene transcription and the global inflammatory response. Even in scenarios where pathogens encode multiple effectors to target one pathway, knockout of just the protease, as in the case of NleC, is capable of dramatically changing the NF-κB activation status. Therefore, redundancy in effectors is unable to fully explain the effects observed when incomplete cleavage occurs.

Of note, recent progress in elucidating the mechanisms that control the promoter selectivity and transcriptional specificity of NF-κB provides new insights into this apparent inconsistency between the protease-mediated p65 cleavage and the impact on NF-κB-conferred inflammatory gene expression and inflammatory response in host cells. This is best exemplified by our recent study on how the afore-mentioned protease effector NleC interferes with the NF-κB 'specifier' component RPS3 and the selective activation of RPS3-dependent, NF-κB target gene transcription during EPEC and *C. rodentium* infections by cleaving p65 (Hodgson et al., 2015). NleC was recently revealed as an important effector functioning to dampen the secretion of inflammatory cytokines in A/E pathogen-infected cells by cleaving p65 (Baruch et al., 2011, Li et al., 2014, Muhlen et al., 2011, Pearson et al., 2011, Shames & Finlay, 2011, Turco & Sousa, 2014, Yen et al., 2010). Injected via the T3SS into host cells at low abundance, relative to other effector molecules (Deng et al., 2010), NleC cleaves only a small percentage of the p65 molecules in infected host cells, even when ectopically expressed (Baruch et al., 2011, Muhlen et al., 2011, Yen et al., 2010). For many pathogen-encoded proteases it remains uncommon to further characterize the fate and possible function of the cleaved fragments, however, our group uncovered that the newly produced fragments had a further functional role within the cell.

The N-terminal fragment of p65, termed as $p65^{1-38}$ (Hodgson et al., 2015), generated by NleC cleavage, executes a unique function by interfering with the cytoplasmic RPS3/p65 interaction and inhibiting the nuclear translocation of RPS3, but not the remaining uncleaved p65. It is noteworthy that RPS3 occurs along with p65 and IκBα in a portion, but not all, of inhibitory NF-κB complexes in the cytoplasm (unpublished observation) and that the nuclear translocation of RPS3 is crucial for the transactivation of the RPS3/p65-dependent NF-κB target genes (Gao & Hardwidge, 2011, Gao et al., 2009, Sen et al., 2012, Wan et al., 2007, Wan et al., 2011, Wier *et al.*, 2012). By interfering with and preventing RPS3 from nuclear translocation with the cleaved $p65^{1-38}$ fragment, the NleC protease is not required to cleave all or even most of the p65 molecules present within the cell, since disrupting the limited NF-κB complexes containing both RPS3 and p65 is sufficient to alter the RPS3-dependent NF-κB gene transcription (Hodgson et al., 2015, Sen et al., 2012, Wan et al., 2007, Wier et al., 2012). It should be noted that aside from the NleC cleaved $p65^{1-38}$, other effectors introduced into the host cell during infections will limit the amount of active p65 and NleH will also specifically target p65/RPS3, creating redundancy. The RPS3-conferred NF-κB promoter selectivity provides a novel avenue for pathogen interference, effectively "amplifying" the effect of cleaving a small percentage of p65 in A/E pathogen-infected host cells in order to sequester RPS3 in the cytoplasm (**Figure 1**). In support of the interrupted RPS3/p65 model, our previous proof-of-concept study demonstrated that an ectopically expressed N-terminal truncation of p65 is capable of blocking the RPS3/p65 interaction and altering NF-κB activity (Wier et al., 2012). Moreover, this work demonstrated that a host Caspase-3 cleaved $p65^{1-97}$ fragment also functions to block the RPS3/p65 interaction. The power of amplifying the effects of NleC lies in the documented importance of the RPS3/p65-mediated NF-κB signaling for host defense against A/E pathogen infections (Gao et al., 2009, Hodgson et al., 2015, Holmes *et al.*, 2012, Perkins, 2012, Pham et al., 2013, Pham *et al.*, 2012, Wan et al., 2007, Wan et al., 2011). In comparison to the global model, this selective inhibition strategy proposes a more targeted strategy for a pathogen to exert a robust impact on host signaling pathways and inflammatory response with one virulence factor, and the NleC-mediated p65 cleavage

serves as a biologically relevant example of this amplification mechanism, though it is likely not the only scenario where this amplification strategy occurs.

Although not extensively examined in the context of other protease-mediated p65 cleavage, the RPS3-dependent selective inhibition mechanism could also be implicated during other pathogenic infections, particularly when proteases create N-terminal fragments that maintain the p65/RPS3 binding interaction. The sulfahydration modification on the C38 of p65 mediates and stabilizes the interaction with RPS3 (Perkins, 2012, Sen et al., 2012); in this way, p65 fragments could maintain their affinity for RPS3 as long as that modification is preserved. The homology between AIP56 and NleC and the similar cleavage site at the N-terminus of p65 indicate that *Phdp* could employ a similar strategy to suppress host inflammatory response (Silva et al., 2013). Moreover, AIP56 also inhibited the gene expression of certain anti-apoptotic genes, which have been revealed to rely on RPS3/p65 for their transcriptional activation, such as *Birc3* (encoding cellular inhibitor of apoptosis protein-2, cIAP2) and *Bcl2l1* (encoding B-cell lymphoma-extra large, Bcl-XL) (Sen et al., 2012). Hence, it would be logical to hypothesize that the p65 cleavage product produced by AIP56 is also able to maintain its interaction with RPS3, sequestering RPS3 away from full-length p65 in immune cells, as demonstrated in *C. rodentium*-infected colon epithelial cells (Hodgson et al., 2015). As demonstrated in the *in vitro* experiments (Hodgson et al., 2015), the interaction between the AIP56-cleaved p65 fragment and RPS3 would prevent the nuclear accumulation of RPS3. Such an interaction in *Phdp*-infected neutrophils and macrophages would still need to be confirmed with further experiments, as there may be cell type specific effects and the importance of RPS3/p65 signaling has yet to be confirmed in these cells. Moreover, the p65ΔC fragment generated by 3C protease cleavage during poliovirus, ECHO-1, and rhino-14 infections (Neznanov et al., 2005) and the p65 N-terminal fragment generated by the activated host Caspase-3 during HIV infection (Coiras et al., 2008), the *Chlamydia* CT441 protease (Lad et al., 2007a), and the *Leishmania* GP63 protease (Dasgupta et al., 2003, Gregory et al., 2008a) could be capable of blocking the RPS3-p65 interaction, although the fate or function of these N-terminal fragments of p65 have not been well defined yet

with regards to RPS3. In particular, we demonstrated previously that truncated p65 fragments as large as 21-186 (Wier et al., 2012) and 1-311 (Wan et al., 2007) are capable of maintaining their binding to RPS3. Though the most profound RPS3 interference was observed with the $p65^{21-186}$ fragment, the ability of additional larger N-terminal p65 fragments to interfere with the RPS3/p65 interaction and function remains to be elucidated (Wier et al., 2012). Moreover, Johannes *et al.* speculated at the possibility of p65 cleavage occurring to block the anti-apoptotic response during picornavirus infection, noting that some NF-κB target genes, *e.g.* IL-6 and IL-8 (Johannes *et al.*, 1999), appeared unaffected while others, including IκBα, were never reset to the steady state levels (Neznanov et al., 2005). Overall this suggests that p65 cleavage modulates the transcriptional specificity of NF-κB to some degree during these infections. While the entire repertoire of RPS3/p65 dependent genes in distinct cell type(s) has not yet been defined, exploiting systems that show divergent activation and expression of NF-κB target genes will further define this cohort of genes or identify other specifier proteins. The evidence that not only NleC but also other pathogen-encoded proteases generate N-terminal p65 fragments with potential interactions with RPS3 indicates that p65 fragment interference could be a widespread virulence strategy for pathogens in order to abrogate RPS3-dependent NF-κB activation in host cells. It is therefore worthy to further examine whether the cleaved p65 fragments manipulate RPS3, or other specifier proteins, for inhibition or activation of a subset of NF-κB target genes in future studies, in particular when the pathogen-encoded proteases are unable to cleave the majority of the cellular pool of p65. Moreover, when cleavage of a target protein is recognized within host cells, deeper consideration of how the pathogen amplifies its efforts may uncover previously underappreciated protein interactions and gene regulation strategies.

3.6 Conclusion

Emerging evidence highlights pathogen-encoded proteases as critical virulence factors that can have dramatic effects on the course of the infection and the ability of the host to detect and respond to infection. In this review, we have discussed how targeting the NF-κB signaling pathway, specifically

protease-mediated cleavage of p65, has allowed various pathogens to achieve control over their microenvironment. Future studies to characterize proteases would benefit from overcoming some major obstacles, such as 1) confirming their interactions with target proteins *in vivo* as opposed to exclusively relying on ectopic or recombinant expression systems; 2) the development of a unified system to measure protease activity would allow for greater control, consistency, and comparison between different research groups; and 3) the development of more sensitive detection methods to study the spatial-temporal activity, localization, and induction of pathogen-encoded proteases (and other virulence factors) during infections. It is likely that recombinant and overexpression systems may lead to the over-identification of target substrates as they rely on purified proteins mixed at concentrations that may not be biologically relevant. Moreover, interactions detected *in vitro* may not be relevant in the context of *in vivo* infection as these proteins may not function in proximity of each other for the cleavage to be pathophysiologically relevant. The zinc metalloprotease NleC is an example of such a situation, where multiple groups, including our own, have reported differences in the target substrates. While most of the groups employed an NleC ectopic expression system (Baruch et al., 2011, Muhlen et al., 2011, Yen et al., 2010) or purified NleC recombinant proteins (Li et al., 2014, Turco & Sousa, 2014), our group was unable to detect cleavage of targets outside of p65 when examining endogenous protein levels (Hodgson et al., 2015). A similar discussion has occurred in reference to the *Chlamydial* proteases CT441 (Lad et al., 2007a, Lad et al., 2007b) and CPAF (Christian *et al.*, 2010, Zhong, 2011), further demonstrating the need to resolve the difference in proposed target substrates. To further help clarify the results between research groups, a standard unit of protease activity would work in much the same way to unify the results from multiple studies. Variations in the methods and buffers employed for protein purification may account for the discrepancy in substrate specificity and cleavage efficiency reported by different groups. The field would arguably benefit the most by the improvement of the tools used to study the spatial-temporal regulation of virulence factors and their functions *in vivo*. Novel methods for more sensitive detection

of low abundant proteins and their interacting partners within host cells would allow for a deeper understanding of how pathogens manipulate host cell signaling pathways.

Determining the fate of cleaved fragments generated by pathogen-encoded proteases or host proteases to elucidate any further activity would also significantly benefit the field, as exampled by the NleC and *Chlamydia* CT441 studies (Hodgson et al., 2015, Lad et al., 2007a, Lad et al., 2007b). The targeting and cleavage of host proteins is an advantage that pathogens have acquired, enhancing their odds of survival. We have highlighted events in which this advantage has resulted in the regulation of a cellular pathway in a very precise and particular manner, which would have gone unnoticed without the full characterization of the cleaved fragments role in the host cell. RPS3 selective targeting serves as a mechanism by which aspects of a signaling hub, where multiple pathways converge, are targeted without disrupting the entirety of hub activity (Brodsky & Medzhitov, 2009). By targeting the RPS3 branch, pathogens are specifically blocking inflammatory and anti-apoptotic signaling within host cells. Other NF-κB specifiers have already been identified, such as Sam68 (Fu et al., 2013) and KLF6 (Zhang et al., 2014), and their functions could also serve as targets for selective inhibition by pathogens in a cell type specific manner. Our model demonstrates that as the promoter selectivity and specificity of NF-κB regulation is further elucidated, there are opportunities to uncover further virulence strategies by pathogens that have been until now underappreciated. Advancements in the tools available and a deeper understanding of the cell types and tissues where specifier proteins are playing a role in regulating NF-κB target genes would also add significantly to the study of host-pathogen interactions. The development of conventional and/or conditional knockout mice lacking these specifier proteins would provide further insight as to the importance of infection site (target cell type), pathogen-mediated blockade of a signaling pathway, and the effect this has on the subsequent host responses to infections. As discussed briefly above, the hypothesis that anti-apoptotic responses dominate the tissue response, whereas survival and inflammatory responses dominate the infiltrating immune cell response can be teased apart through the creation and use of lineage specific knockout mice (*i.e.* conditional RPS3 ablation driven

by the Villin-Cre transgene to knockout RPS3 in intestinal epithelial cells). Such innovations would clarify the crosstalk and redundancy among virulence factors, opening the field for the development of more targeted and specific therapeutics to combat pathogens.

3.7 Acknowledgements

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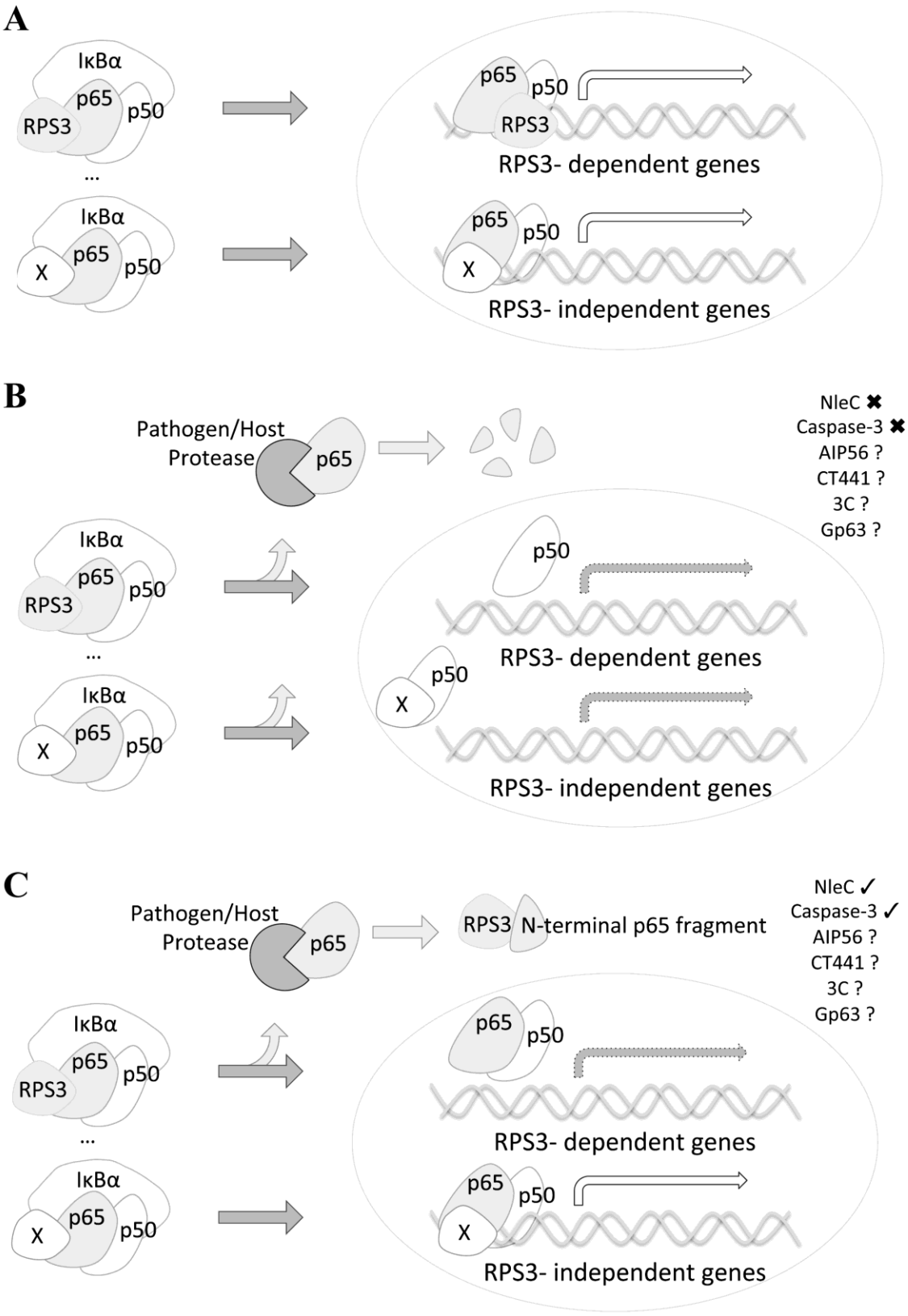
3.8 Figure

Figure 3.1 Comparison of linear and selective inhibition interpretation models of pathogen-protease mediated NF-κB interference.

Figure 1. Comparison of global and selective inhibition interpretation models of pathogen-encoded protease mediated NF-κB interference. Under infection conditions, NF-κB activation results in p65 nuclear translocation and the transactivation of a wide array of target genes, a subset of which require the aid of known specifier proteins, such as RPS3 and Sam68. This leads to a coordinated cellular responses and clearance of infection (A). Pathogen-encoded proteases can have devastating, widespread effects (global, B) or they can inhibit specific aspects of a pathway. Complete cleavage of p65 interrupts the signal transduction cascade, preventing all downstream outcomes in a global fashion. However, partial proteolytic processing of p65 can result in selective inhibition (C), as there are still some full length and functioning p65 molecules within the cell. In the experimentally verified (checked) examples of NleC-mediated cleavage of p65, during A/E pathogen infections, an N-terminal fragment is produced that sequesters RPS3, preventing the activation of that arm of the NF-κB signaling pathway. This inhibition thus results in the amplification of the effect of cleaving only a percentage of the p65 cellular pool. It is likely, yet remains to be verified experimentally (question marked), that a similar mechanism is employed by other pathogens. EPEC/EHEC/*C. rodentium*- NleC; *Phdp*- AIP56; *C. trachomatis*- CT441; Poliovirus- 3C; *Leishmania spp.*- Gp63; Host- Caspase-3.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3.9 Tables

Table 3.1 Pathogen-encoded and co-opted proteases targeting other NF-κB signaling molecules

Pathogen	Protease	Protease category	Host target	Cleavage site(s)	Cleavage verification	Reference
EPEC	NleC	Zinc Metalloprotease	p50	Not characterized	*in vitro*	Pearson *et al.* 2011
EPEC	NleC	Zinc Metalloprotease	p50	Not characterized	*in vitro*	Muhlen *et al.* 2011
EHEC	NleC	Zinc Metalloprotease	RelB	C144/E145	recombinant	Turco & Sousa 2014
EHEC	NleC	Zinc Metalloprotease	p50	C61/E62	recombinant	Turco & Sousa 2014
CVB3	$3C^{pro}$	Serine Protease	IκB□	Q249/G250	recombinant, *in vitro*	Zaragoza *et al.* 2006
HCV	NS3/4A	Serine Protease	CARDIF/ MAVS	C508/H509	*in vitro*	Meylan *et al.* 2005; Li *et al.* 2005b
HCV	NS3/4A	Serine Protease	TRIF	C372/S373	recombinant, *in vitro*	Li *et al.* 2005a
EV68	3C	Serine Protease	TRIF	Q312/S313, Q653/S654	*in vitro*	Xiang *et al.* 2014
PRRSV	NSP4	Serine Protease	NEMO	E349/S350	*in vitro*	Huang *et al.* 2014
Y. enterocolitica	Caspase-3 (host)	Cysteine Protease	MyD88	D135/S136	recombinant, *in vitro*	Novikova *et al.* 2014

EPEC, Enteropathogenic *E. coli*; EHEC, Enterohemorrhagic *E. coli;* CVB3, Coxsackievirus B3; HCV, Hepatitis C virus; EV68, Enterovirus 68; PRRSV, Porcine reproductive and respiratory syndrome virus; *Y. enterocolitica, Yersini enterocolitica;* recombinant, purified protein assays; *in vitro*, cell culture/ transfection assays; *in vivo,* animal studies.

Table 3.2 Pathogen-encoded and co-opted proteases targeting p65

Pathogen	Protease	Protease category	p65 cleavage site(s)	Cleavage verification	Fate and function of p65 fragment	Proposed mechanism for NF-κB inhibition	Reference
EPEC, EHEC	NleC	Zinc Metalloprotease	P10/A11	*in vitro*	Undetermined	Linear	Yen *et al.* 2011
EPEC	NleC	Zinc Metalloprotease	Not characterized	*in vitro*	Undetermined	Linear	Muhlen *et al.* 2011
EPEC	NleC	Zinc Metalloprotease	C38/E39	*in vitro*	Undetermined	Linear	Pearson *et al.* 2011
EPEC	NleC	Zinc Metalloprotease	C38/E39	recombinant *in vitro*	Undetermined	Linear	Baruch *et al.* 2011
EPEC, *C. rodentium*	NleC	Zinc Metalloprotease	Not characterized	*in vitro*, *in vivo*	Undetermined	Linear	Sham *et al.* 2011
EHEC	NleC	Zinc Metalloprotease	C38/E39	recombinant	Undetermined	Undetermined	Li *et al.* 2011
EHEC	NleC	Zinc Metalloprotease	C38/E39	recombinant	Undetermined	Undetermined	Turco & Sousa 2014
EPEC, *C. rodentium*	NleC	Zinc Metalloprotease	P10/A11, C38/E39	*in vitro*, *in vivo*	N-terminal fragment binds RPS3	Amplification	Hodgson *et al.* 2015
Phdp	AIP56	Zinc Metalloprotease	C39/E40 (fish p65)	recombinant *in vitro*	Undetermined	Linear	Silva *et al.* 2015
C. trachomatis	CT441	TSP Protease	F351/T352	*in vitro*	p40 product binds to IκBα	Amplification	Lad *et al.* 2007a; Lad *et al.* 2007b
Leishmania	Gp63	Metalloprotease	C-terminus	*in vitro*	p35 fragment binds DNA with p50	Amplification	Gregory *et al.* 2008a
Poliovirus	3C	Cysteine Protease	Q480/G481	*in vitro*	Undetermined	Linear	Neznanov *et al.* 2005
HIV-1	Caspase-3 (host)	Cysteine Protease	D97/G98	recombinant *in vitro*	binds p50/IκBα	Amplification	Coiras *et al.* 2008

EPEC, Enteropathogenic *E. coli*; EHEC, Enterohemorrhagic *E. coli; Phdp, Photobacterium damselae piscicida*; *C. trachomatis, Chlamydia trachomatis;* HIV, human immunodeficiency virus type 1; recombinant, purified protein assays; *in vitro*, cell culture/ transfection assays; *in vivo,* animal studies.

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4 Identification and characterization of a *C. rodentium* mutant strain with attenuated virulence in IL-22$^{-/-}$ mice

4.1 Introduction

Gastrointestinal infections remain a leading cause of morbidity and mortality worldwide that causes a serious economic and public health burden (Clements et al., 2012; Donnenberg and Whittam, 2001; Hartland and Leong, 2013). Attaching and effacing (A/E) pathogen infections, such as Enteropathogenic and Enterohemorrhagic *E. coli*, contribute significantly to this burden (Ochoa and Contreras, 2011). The mouse specific pathogen, *Citrobacter rodentium*, has served as a model for the study of A/E pathogens and has been instrumental in the discovery and characterization of novel aspects of gut immunology (Borenshtein et al., 2008; Collins et al., 2014; Koroleva et al., 2015; Mundy et al., 2005). Upon infection with *C. rodentium*, mice experience acute self-limiting colitis that clears approximately 4 weeks post infection. During this phase, there is a considerable proinflammatory cytokine response elicited by immune cells responding to infection. Interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 6 (IL-6), and IL-22 are all induced during infection and act on the epithelial and immune cells to clear the infection (Collins et al., 2014; Dann et al., 2014; Li et al., 2014; Symonds et al., 2009; Zheng et al., 2008). The role of IL-22 during *C. rodentium* infection has recently garnered attention for bridging the gap between the epithelial innate immune response and the lymphocytic driven adaptive response to infection (Eidenschenk et al., 2014; Honda, 2012; Rutz et al., 2013; Rutz et al., 2014).

Produced only by immune cells, both adaptive CD4 T cells and innate lymphocytes (ILCs), IL-22 signals to cells expressing its cognate receptor, IL-22R1 and IL-10R2, which are primarily epithelial cells (Basu et al., 2012; Collins et al., 2014; Eidenschenk et al., 2014; Kumar et al., 2013; Sabat et al., 2014). In the gut, IL-22 acts on colon epithelial cells and goblet cells to induce the production of antimicrobial peptides such as RegIIIγ, RegIIIβ, S100A8, and mucin (Collins et al., 2014; Zenewicz et al., 2013; Zheng et al., 2008). Particularly, the production of IL-22 by ILCs at early stages has been credited with the early

control of bacterial proliferation, functioning to limit the bacterial burden (Cella et al., 2009; Satoh-Takayama et al., 2009; Zheng et al., 2008). The CD4 Th22 response takes over the production of IL-22 at later stages of infection to amplify the anti-microbial peptide expression (Basu 2012). In the IL-22$^{-/-}$ setting, *C. rodentium* infection leads to severe morbidity and mortality within the first two weeks of infection. This is accompanied by a break in the barrier integrity, leading to the dissemination of bacteria systemically, presumably contributing to mortality (Aychek et al., 2015; Zheng et al., 2008).

To further understand the key host-pathogen interactions during *C. rodentium* infection in the presence and absence of IL-22, we sought to identify an attenuated strain of *C. rodentium* that would allow deeper characterization of the effects of IL-22. The use of an attenuated strain of *C. rodentium* would prevent the rapid decline toward death, providing more opportunity to study the effects of IL-22 at all stages of the immune response. From our stock of *C. rodentium* strains, we endeavored to screen them using IL-22$^{-/-}$ mice. Upon considering strains for screening, we opted not to use strains that were known not to cause infection in wild type mice. We also did not use strains that harbor mutations that induce a more robust inflammatory response than wild-type *C. rodentium* for fear that this would exacerbate mortality witnessed in IL-22$^{-/-}$ mice. We determined that the Mut1 strain would be a worthy candidate for characterization in IL-22$^{-/-}$ mice as we were unaware of the mutation it harbored but also saw no robust changes in infection in wild-type mice previously. Here we present the initial characterization of the Mut1 strain in infection of IL-22$^{-/-}$ as well as the identification of the gene disrupted in the Mut1 strain of *C. rodentium*.

4.2 Results

4.2.1 Infection with Mut1 causes less severe morbidity and mortality in IL-22$^{-/-}$ mice

C. rodentium infection in IL-22$^{-/-}$ mice results in a severe phenotype in which only 20% of mice survive by day 14, compared to a 100% survival rate in wild-type mice (Zheng et al., 2008). Therefore, IL-22$^{-/-}$ mice are an ideal tool for screening and characterizing mutant strains of *C. rodentium* for attenuated

phenotypes that may not be as competitive in wild-type hosts. Using these mice, we screened and characterized a mutant *C. rodentium* strain entitled Mut1 for its ability to cause infection and inflammation in IL-22$^{-/-}$ mice. At early time-points Mut1-infected animals have similar weight loss (Figure 4.1A) and clinical scores (Figure 4.1B) as wild-type *C. rodentium* infected mice. However, after day 8-post infection the weight loss between the two groups consistently differed by approximately 10% (Figure 4.1A). Also at this time point, the Mut1-infected animals clinical symptoms begin to improve, as determined by a reduction in the clinical score (Figure 4.1B), whereas the wild-type *C. rodentium* infected animals continue to deteriorate. Consistently, Mut1 infected animals experience 80% survival following infection; compared to the observed 20% survival in wild-type *C. rodentium* infected animals (Figure 4.1C). Therefore, the Mut1 strain causes an attenuated infection compared to the effects of wild-type *C. rodentium* infection in IL-22$^{-/-}$ mice.

4.2.2 Mut1 C. rodentium displays no defects in growth or attachment to CEC

We examined the growth kinetics of the Mut1 strain to determine the cause of the attenuation. First, we established that there was no difference in the replication *in vitro* when Mut1 and wild-type *C. rodentium* were grown in LB broth (Figure 4.2A). Second, we assessed the ability of the Mut1 strain to attach to colon epithelial cells (CEC) isolated from IL-22$^{-/-}$ mice *in vitro*. There was no difference in bacterial attachment between the Mut1 and wild-type *C. rodentium* strains (Figure 4.2B) suggesting that Mut1 has no defect in forming attachments to CEC and therefore establishing infections. This suggests that the bacterial machinery used for attachment is unaffected by the Mut1 mutation.

To determine the Mut1 growth and attachment *in vivo,* we examined the fecal clearance from IL-22$^{-/-}$ animals following infection with either strain. There was no difference in the bacterial load between the Mut1 and wild-type *C. rodentium* infected animals (Figure 4.2C), indicating that the Mut1 strain is capable in replicating and maintaining itself within the IL22$^{-/-}$ gut. In order to assess the *in vivo* attachment

of the Mut1 strain, we harvested the colons of mice at 8 days post infection, and stained for LPS in paraffin embedded sections. Day 8 was chosen as the time-point just before the wild-type *C. rodentium* infected mice began to experience significant morbidity and mortality. Almost no LPS staining was observed in the colons from Mut1 infected animals except for in small areas along the top of the crypts (Figure 4.2D second panel), however positive LPS staining was readily detected along almost the entire length of the colons sections of wild-type *C. rodentium* infected mice (Figure 4.2D third panel). In some areas dramatic infiltration of the bacteria into the colonic crypts is visible (Figure 4.2D last panel), as reported previously (Zheng et al., 2008). Taken together, these results indicate that Mut1 and wild-type *C. rodentium* strains are replicating at the same rate *in vivo* albeit in different locations.

4.2.3 Animals infected with Mut1 infection display limited intestinal inflammation

Next, we wanted to characterize the inflammatory response to Mut1 infection at the same day 8-time point. Histological analyses of Mut1-infected IL22$^{-/-}$ mice demonstrated minimal signs of inflammation; in striking contrast, the wild-type *C. rodentium* group displayed immune cell infiltration, thickening of the colonic crypts, goblet cell loss, and hyperplasia (Figure 4.3A). The lack of bacterial colonization of epithelial cell layer is visible here. Similar to Figure 4.2D, wild-type *C. rodentium* is clearly visible in focal aggregates and microcolonies (Bergstrom et al., 2010) over the epithelial cells and into the colonic crypts at this time-point.

We then examined a panel of genes to characterize the inflammatory response. Target genes were chosen for the known or suggested roles during wild-type *C. rodentium* infection in wild-type mice. Infection with Mut1 did not induce the expression of *Cxcl1*, *Cxcl2*, *Cxcl5*, *Nos2*, *Il6*, *Ifng*, *Il17a*, and *Il2* (Figure 4.3B) and in some cases suppressed their expression when compared to wild-type *C. rodentium*. There was also no up-regulation of matrix metalloprotease 9 (*Mmp9*) and *Mmp10* in Mut1-infected animals as was seen in the wild-type *C. rodentium* infected animals, consistent with a lack of inflammatory

environment (Koller et al., 2012; Liu et al., 2013) caused by Mut1 infection (Figure 4.3B and Figure 4.4). The induced expression of *Cxcl1*, *Cxcl2*, and *Cxcl5* typically leads to the recruitment of neutrophils, which are a major source of IL-22 during infection (Brown et al., 2011; Crepin et al., 2015; Lee et al., 2015; Shea-Donohue et al., 2008; Spehlmann et al., 2009). Along with these chemokines, the induction of *Mmp9*, *Mmp10*, and *Icam1* also allow infiltration of immune cells into the colon tissue following wild-type *C. rodentium* infection. At this time point we did not observe the robust induction of *Il4*, *Il10*, *Il12*, *Il21*, or *Il23* by either strains, as they are likely induced at a later time-point.

4.2.4 Mut1 infection does not induce barrier disruption or systemic bacterial dissemination

Taking together the induction of matrix metalloproteases, the robust proinflammatory response, and previous work demonstrating systemic spread of wild-type *C. rodentium* in IL-22$^{-/-}$ mice (Zheng et al., 2008), we examined the effect of infection on tight junctions in the epithelial cell layer as an indicator of barrier function. The regulation of barrier permeability relies upon the maintenance of tight junctions between the epithelial cells along the gastrointestinal tract, as they regulate the passage of ions and metabolites (Conlin et al., 2009). Colon sections from day 8-post infection were examined for immunofluorescence staining of claudin-3, a tight junction protein that has previously shown to be altered by wild-type *C. rodentium* infection (Conlin et al., 2009). Mut1-infected animals did not appear to have significant changes in the claudin3 staining, where there was intense and distinct cell surface staining (Figure 4.5A). Conversely, wild-type *C. rodentium* infected animals appeared to have diffuse and low-intensity staining, indicative of tight junction disruption (Figure 4.5A). Consistent with the maintenance of claudin-3 (Figure 4.5A), Mut1 infected animals did not have recoverable, live bacteria in their spleens or livers (Figure 4.5B). However, the spleens and livers of wild-type *C. rodentium* infected animals had a robust amount of bacteria present (Figure 4.5B). The overall lack of a response to infection with Mut1 *C. rodentium* suggests that the genetic deletion has abrogated a key virulence factor that has altered many aspects of the typical course of disease in IL-22$^{-/-}$ mice. While the Mut1 strain has no defect in the ability

to colonize and maintain itself within the host, it is also not causing any immune activation or even serious clinical symptoms possibly due to its lack of interaction with the epithelial cell layer.

4.2.5 Mut1 carries a disruption of a putative DNA recombinase

Random transposon mutagenesis is a powerful genetic tool used for the screening of bacterial genes by randomly interrupting a coding region via the insertion of a transposable element and, typically, a resistance marker (Serina et al., 2004; Xu et al., 2011). Using the sequence of the transposable element and taking advantage of two particular 5 nucleotide sequences (termed anchor sequences) that repeat approximately every 245 or 252 basepairs of the genome, we devised a PCR strategy to selectively amplify the genomic sequence upstream of the transposable element (Figure 4.6A-B). After we amplified the target region, sequencing and BLAST searches of the wild-type *C. rodentium* genome were conducted to determine the site of insertion by sequence alignment. The Mut1 strain contained an interruption in a site-specific DNA recombinase that has yet to be characterized (Figure 4.6C). The presence of the transposable element at this location was confirmed by PCR using primers specific to the wild type genomic sequence at the 5' and 3' ends of the recombinase gene (set 1), as well as by primers specific to the 5' end of the gene and the transposon (set 2) (Figure 4.6C). Using set 1 primers for the recombinase gene, the wild-type *C. rodentium* and Δ*EscN* strain (Wan et al., 2011) (used as an additional negative control), show bands corresponding to the size of the recombinase gene, approximately 600 kb (Figure 4.6D). There is no detectable band for the Mut1 gene as expected, given the extension times used for the reaction and the expected size of the gene and the transposable element that interrupts the gene. To further confirm the location of the transposable element, we used primers for set 2 that cover the 5' part of the gene and the 5' end of the transposon. In these reactions, only the Mut1 strain has a detectable band, as the wild-type *C. rodentium* and Δ*EscN* strain have no transposable element. Further characterization of this recombinase gene is required to determine if this is responsible for the defective/attenuated infection phenotype caused by the Mut1 strain.

4.3 Discussion and Future Directions

The importance of IL-22 for maintaining and reinforcing the epithelial barrier in the gut is underscored by the severe morbidity and mortality experienced by IL-22$^{-/-}$ mice following *C. rodentium* infection (Basu et al., 2012; Honda, 2012; Pickert et al., 2009; Zheng et al., 2008). Herein, we demonstrated the use of IL-22$^{-/-}$ mice as a tool to screen mutant strains of *C. rodentium* for altered phenotypes, which may lead to the identification and characterization of novel virulence factors that will likely go undetected using wild-type mice. Currently, there have been many reports describing the importance of a particular gene important for infection (by the pathogen) or for protection from infection (by the host). We consider the combination of these strategies to provide a larger window for the detection of valuable host-pathogen interactions. Hence, we employed the use of IL-22$^{-/-}$ mice, as their increased susceptibility to infection should render them an ideal setting to rapidly screen many strains and observe attenuated *C. rodentium* strains. However, many further experiments are needed to fully elucidate the importance of the DNA recombinase for *C. rodentium* infection and the exact role that IL-22 is playing in protecting against fatal outcomes of infection.

4.3.1 Mut1 replicates within the lumen and not at the epithelial cell layer

The modest morbidity caused by Mut1 infection in IL-22$^{-/-}$ mice suggests that the strain is deficient in its ability to colonize the epithelial cell layer directly *in vivo* albeit maintaining the ability to replicate and attach to individual epithelial cells *in vitro*. The physical separation of the Mut1 bacteria from the epithelial cells is consistent with the lack of response to infection, as the degree of bacterial penetration into the tissue has been previously linked to the degree of inflammatory response (Assi et al., 2013; Johansson et al., 2014; Reid-Yu et al., 2015; Yoshida et al., 2006). Mutant strains of *C. rodentium* harboring severe defects in the major virulence genes, such as those without a functional type three secretion system (T3SS) or missing the regulator *Ler,* are completely unable to maintain themselves in the gut and immediately cleared (Bergstrom et al., 2008; Bergstrom et al., 2010; Kamada et al., 2012). These

strains are never detectable in the fecal pellets from infected mice, unlike the Mut1 strain that is readily detectable in the feces up to at least 19 days post infection.

As the fecal clearance data do not show differences between the Mut1 and wild-type *C. rodentium* strains, this may not be the best measure to determine infection rates. Indeed, the number of bacteria present in the fecal samples and the lumen is not always reflective of the number of bacteria that actually infect epithelial cells (Bergstrom et al., 2010; Bhullar et al., 2015; Hansson, 2012; Johansson et al., 2011).Therefore further experiments are need to determine the quantity of bacteria attached to the tissue compared to that found within the lumen to provide a more accurate picture of the infection rates by Mut1 and wild-type *C. rodentium*. Colon tissues collected from infected mice at various time points should be separated into the luminal contents and the tissue. After extensively washing the tissue, both the luminal contents and the tissue should be serially diluted and plated to determine the CFU present in each compartment of the colon. Both Mut1 and wild-type *C. rodentium* infected animals will likely demonstrate a bacterial burden within the luminal contents that increases until peak of infection and then wanes. It has been previously established that as *C. rodentium* infection reaches peak bacterial loads in the fecal pellets, the bacteria transition from presiding primarily in the lumen to increasing their presence within the inner mucus layer, gaining closer contact with the epithelial cells (Gustafsson et al., 2013). Hence, there should be an expected increase in the number of tissue-associated bacteria as the time points approach peak infection roughly 10-14 days post infection. The lumen to tissue transition is likely not occurring, or occurring in a very limited capacity, in Mut1 infected animals, given the previously demonstrated lack of LPS staining at the tissue and the limited inflammatory profile. However, wild-type *C. rodentium* infected animals will likely demonstrate tissue-associated bacterial burdens that increase over time as the animals approach peak infection or a moribund state. This increase in bacterial attachment to the epithelial cells will correspond to the inflammatory profile previously discussed. If the Mut1 strain is primarily residing within the lumen and not reaching the epithelial cells, it suggests that the genetic defect of Mut1 does not completely prevent infection, yet alters the colonization location away from the epithelial cell layer, abolishing the pathogen's ability to maintain its desired niche.

4.3.2 IL-22$^{-/-}$ mice have an altered mucus composition

Further examination of the entire luminal structure of the colon of IL-22$^{-/-}$ mice is essential for our understanding of role IL-22$^{-/-}$ is playing in protecting the colon. The entire tract of the colon is lined with a mucus layer that serves as both a mechanical and chemical means to shield the epithelial cells from the microbiota and luminal contents. The outer mucus layer is heavily colonized with commensal bacteria (Johansson et al., 2011) where the mucin glycoproteins serve as both scaffold and an energy source for bacteria (Bergstrom et al., 2010; Johansson et al., 2011; Lievin-Le Moal and Servin, 2006; Mizoguchi, 2012). However, the inner layer is more complex and primarily sterile, unless virulence factors allow invading bacteria to penetrate the inner mucus layer (Bergstrom et al., 2010; Hansson, 2012; Johansson et al., 2014; Lievin-Le Moal and Servin, 2006). IL-22 has been demonstrated to enhance the production of mucus and induces goblet cell restitution following inflammation (Sugimoto et al., 2008). The IL-22-STAT3 signaling pathway activates mucin expression in goblet cells as well as induces the expression anti-microbial peptides (Lievin-Le Moal and Servin, 2006; Mizoguchi, 2012; Sugimoto et al., 2008). Moreover, anti-IL-22 treatment in mice abolishes the mucus layer (Sugimoto et al., 2008), yet the effect of genetic ablation of IL-22 on the mucus layer has yet to be directly examined. This point is especially important when considering that IL-22$^{-/-}$ mice have normal colonic architecture, displaying no overt spontaneous colitis (Zenewicz and Flavell, 2011; Zenewicz et al., 2013). This may be in part because until now IL-22 has been implicated in inducing the expression of some mucin proteins (Sugimoto et al., 2008), but not Muc2, which is the primary structural protein of the two mucus layers found in the colon (Johansson et al., 2011; Mack et al., 1999).

Hence, future experiments should include the isolation of colons from IL-22$^{-/-}$ mice using Carnoy fixative to preserve the entire luminal structure (Cohen et al., 2012; Johansson et al., 2011). Tissue sections from these colons should be stained for the various mucin proteins in order to determine the mucus composition in uninfected IL-22$^{-/-}$ mice as a baseline. It is likely that IL-22$^{-/-}$ mice have some degree of a Muc2-containing mucus layer, considering that Muc2$^{-/-}$ mice develop spontaneous colitis

(Johansson et al., 2011; Van der Sluis et al., 2006), which is not seen in IL-22$^{-/-}$ mice as mentioned previously. These sections from uninfected animals can be compared with age matched Mut1 and wild-type *C. rodentium* infected colons processed in the same manner. This will inform the effect of Mut1 and wild-type *C. rodentium* on mucin production during infection as well as confirm the compartment of the colon colonized by each strain of *C. rodentium,* with the addition of LPS staining. Typically during inflammation and infection, mucin production is induced to flush out the invading pathogen as an innate mechanism for protecting the host epithelial cell layer. To measure this, the thickness of the mucus can be measured in each of the conditions. Therefore, consistent with the inflammatory profiles already observed, it is likely that Mut1 infection will elicit little to no mucus production, while the wild-type *C. rodentium* strain will induce increased mucus secretion and production. In an effort to confirm these findings, mRNA transcripts for the various mucin proteins can also be analyzed by RT-PCR, where similar results would be expected.

4.3.3 Mut1 is unable to penetrate the colonic mucus layer

During normal infection, *C. rodentium* is able to transition from the lumen to the epithelial cells by penetrating through the mucus layer (Bergstrom et al., 2010; Hansson, 2012). The previous proposed experiments staining for LPS on Carnoy fixed tissue will likely confirm that the Mut1 *C. rodentium* is replicating in either the outer mucus layer or the lumen of the colon and unable to reach the epithelial cells. To directly examine whether the Mut1 strain would be capable of inducing a robust infection in the absence of the mucus layer, Muc2$^{-/-}$ mice should be infected with either Mut1 or wild-type *C. rodentium* and followed for signs of morbidity, such as fecal colonization, weight loss, and survival. If the Mut1 strain is capable of causing infection to a similar degree as the wild-type *C. rodentium* strain in this scenario, then the major default of the Mut1 strain is its inability to bypass the mucus layer. There may a defect in the ability of Mut1 bacteria to survive in the mucus layer. To address this hypothesis, crude mucus from IL-22$^{-/-}$ mice can be extracted from mucosal scrapings of the colon tissue (Bergstrom et al., 2010) and incubated with cultures of Mut1 or wild-type *C. rodentium* at various concentrations. Colony

forming units (CFUs) counted from each strain can be compared after serial dilution to determine the differences in the survival of either strain. The Mut1 strain is likely to survive the mucus exposure *in vitro* similarly to wild-type *C. rodentium* because despite the inability to establish close colonization of the epithelial cell layer, the Mut1 strain is able to find a niche and maintain itself within the lumen of the colon as it is readily detected in the feces for many days post infection.

Alternatively, binding to the mucus layer will also prevent its ability to penetrate through to the epithelial cells. Therefore an ELISA-like-assay (Linden et al., 2008) can be preformed using mucin bound to a microtiter plate and incubated with labeled Mut1 or wild-type *C. rodentium*. The labeled bacteria are produced using standard kits such as BacLight (Life Technologies), which label bacteria with either red or green fluorescent dyes that can be read using a plate reader measuring the appropriate channels. It may be that the Mut1 strain retains its ability to bind to mucin, establishing itself in the outer mucus layer, yet cannot further penetrate to the inner mucus layer since we cannot detect Mut1 bacteria in close association with the epithelial cells *in vivo*.

Upon observing no differences in bacteria survival or mucin binding, it may be that the Mut1 strain is unable to digest its way through the mucosal layer. The importance of the mucus layer described above suggests that invading bacteria need to encode mechanisms that allow penetration of the physical and biochemical barrier posed by the complex mucin proteins. It is currently hypothesized that all gut bacteria employ one of two ways to penetrate the mucus layer, via flagella-mediated motility or via degradation of mucin proteins by proteases (McGuckin et al., 2011). Since *C. rodentium* encodes no flagella, it is likely that it relies on mucinase like proteins for invasion of the mucus layer. As previously mentioned, enteric pathogens encode proteases that allow the digestion of the mucin layers in order to access the epithelial cells (Bhullar et al., 2015; Grys et al., 2005; Grys et al., 2006; Lidell et al., 2006; Moncada et al., 2006). Currently, there are few such proteases characterized in *C. rodentium*, such as the serine protease autotransporter, Pic (Bhullar et al., 2015). The Pic protease has multiple functions, among which is mucinase activity. However, when the Pic gene was disrupted it didn't alter the virulence of the

mutant strain (Bhullar et al., 2015). This suggests that there are other mucinases encoded by *C. rodentium* that could compensate for Pic that remain uncharacterized. Similarly, EHEC encodes a secreted zinc metalloprotease, StcE, targets mucin 7 (MUC7) that may aid in penetrating the mucus layers in the gut upon infection (Grys et al., 2005; Grys et al., 2006). These studies determined that EHEC expressing StcE exhibited increased intimate attachment to cells in culture and mucinase activity *in vitro*. While BLAST searches reveal no homologous protein in *C. rodentium*, further work is needed to identify the virulence genes required for penetrating the gut mucosa.

Without a clear target protein to purify, the mucinase activity of Mut1 and wild-type *C. rodentium* can be measured using the following two assays. First, agar plates can be prepared utilizing mucin as the sole carbon source (Collier et al., 2003; Deplancke et al., 2002; Png et al., 2010) in this way, only bacteria expressing a mucolytic enzyme are capable of accessing the carbon source needed for growth and survival. If Mut1 *C. rodentium* are unable to form colonies on this media and wild-type *C. rodentium* do, it would suggest that Mut1's inability to access the epithelial layer *in vivo* stems from its inability to digest through the mucus layer. Secondly, the supernatants from Mut1 and wild-type *C. rodentium* cultures should be collected and concentrated to collect secreted proteins. These supernatants can then be incubated with mucin, either crude or from a commercial source such the bovine submaxillary mucin from Sigma (Bhullar et al., 2015). After the appropriate incubations, the reactions can be separated on SDS-PAGE gels and analyzed with a glycoprotein staining kit. Digested mucin proteins will run at lower molecular weights, while undigested glycoproteins will remain mostly in the stacking gel, appearing as a smear, due to their high molecular weights.

4.3.4 IL-22 is needed to protect against LPS-induced shock following infection

In our studies, we also examined the barrier permeability of IL-22$^{-/-}$ mice during infection with either Mut1 or wild-type *C. rodentium* by examining the localization of claudin 3, a tight junction protein, and examining the spread of *C. rodentium* to the liver and spleen of infected animals. Given our current understanding, *C. rodentium* infection alters the integrity of the epithelial cell barrier (Conlin et al., 2009;

Guttman and Finlay, 2009) by inducing a redistribution of the tight junction proteins. This results in the dissemination of luminal contents into the bloodstream and conversely the leak of protein rich serum into the lumen. In an IL-6$^{-/-}$ setting, *C. rodentium* infected mice experienced ulcerations that allowed for an influx of nutrients from the bloodstream which gave rise to dense micro-colonies observed intimately associated with the epithelial layer (Dann et al., 2008). Similar ulcerative lesions are observed in Muc2$^{-/-}$ mice during infection (Bergstrom et al., 2010), in IL-22$^{-/-}$ mice during infection (Zheng et al., 2008) (and ourselves), and TCRα deficient mice given a mucolytic drug (Sugimoto et al., 2008). In contrast, SCID mice infected with *C. rodentium* are colonized across the epithelial cell surface without ulceration or micro-colony formation (Dann et al., 2008). These studies support further examination of the effect that infection-induced ulcers and epithelial cell injury have on the severe morbidity and mortality experienced by these mice following infection.

Future studies should measure the degree of barrier permeability following infection with wild-type *C. rodentium* and Mut1 using the FITC-dextran method (Bergstrom et al., 2010; Gibson et al., 2008a; Gibson et al., 2008b). Due to the lack of inflammatory response and cell association of the Mut1 strain, it is unlikely to cause any defect in the barrier integrity. However, given the large bacterial loads recovered from the spleens and livers of wild-type *C. rodentium* infected IL-22$^{-/-}$ mice, it is expected that the FITC-dextran will be readily detected in the blood of these mice during the latter stages of the infection, 7 to 10 days. The presence of bacteria in the blood and at these typically sterile sites is not enough to cause the significant mortality we observed, since there is also a degree of bacterial escape following infection in wild-type mice (Zheng et al., 2008). Moreover, intravenous challenge of wild type mice with *C. rodentium* does not cause mortality (McDaniel et al., 2015). This may be attributed to the protective effect of IL-22 on the liver by inducing the production of LPS binding protein, which has been shown to temper systemic inflammation (Sabat et al., 2014). It would be intriguing to determine if localized delivery of IL-22 to the liver (Sugimoto et al., 2008) or the systemic distribution of LPS binding protein would be sufficient to rescue IL-22$^{-/-}$ from mortality.

4.3.5 Characterizing the function of the putative DNA recombinase disrupted in Mut1

The attenuated phenotype of Mut1 *C. rodentium* suggests that the specific mutation alters its pathogenicity in IL-22$^{-/-}$ mice compared to wild-type *C. rodentium*. Ultimately, we determined that the Mut1 strain carries an interrupted, uncharacterized, site-specific DNA recombinase (Figure 4.6). Confirming the protein product as well as constructing a KO strain specifically for the recombinase gene would help clarify the effect of this gene on pathogenicity. This gene is located within a 5.3 kb long genomic island that has no known function currently (Petty et al., 2010) and shares homology upwards of 50% with various other pathogenic and non-pathogenic enteric bacteria recombinases (BLAST, NCBI). Site-specific recombinases induce one of three changes to the genomic structure of the organism, i.e. integration (as in the classical phage infection), resolution of transposable elements, or inversion of a DNA sequence located between two consensus sequences. There are two major classes of recombinases, tyrosine recombinases and serine recombinases (reviewed in (Grindley et al., 2006)). Based on sequence homology, this recombinase is likely a serine recombinase and therefore may play a role in DNA inversions commonly seen in host adaptations by programmed DNA alterations (Johnson, 2015). Of note, DNA inversions and recombination events are employed by many bacterial pathogens and have been described to play important roles in antigenic rearrangements for host evasion and other virulence mechanisms (Johnson, 2015; Scott et al., 2007).

The on-going reassortment of the genomic material available to pathogens allows for rapid responses from environmental threats as well as explains the diversity of bacterial effectors that even closely related pathogens exhibit (Agbor and McCormick, 2011; Dean, 2011). These reassortment events, either through phage infection or horizontal gene transfer, account for the diversity between EPEC, EHEC, and *C. rodentium* who share just under 3000 genes of the almost 5000 genes encoded by *C. rodentium* (Petty et al., 2010). The homology and shared genes account for the similarity in infection mechanisms and virulence strategies, justifying and supporting the use of *C. rodentium* as a model for EPEC and EHEC infection. Interestingly, Petty and colleagues determined that the genomic flux

observed in *C. rodentium* could be partially attributed to the spontaneous activation and expression of prophages found within the genome under normal growth conditions. Upon examining the activity of these rearrangement events *in vivo*, they determined that in response to host induced stress several prophages might be activated to induce a differential gene expression pattern (Petty et al., 2011). *Campylobacter jejuni* is a prime example of such stress responses. During host infection, there is evidence of frame shifting and inversion of the origin of replication of specific genomic regions to alter gene regulation (Scott et al., 2007). As such, the authors concluded that during infection there are subpopulations of *Campylobacter* expressing various versions of their genome that will allow survival of, at minimum, a subset of these bacteria (Scott et al., 2007).

While it has been demonstrated fairly recently that the *C. rodentium* genome undergoes significant recombination events during infection and passage through the mouse and many of the genomic islands identified are flanked by potential insertion sequences (Petty et al., 2011), our future experiments need to determine whether these recombination events are still occurring in our Mut1 strain. To do this, samples of genomic DNA should be isolated from cultures of wild-type *C. rodentium* and Mut1 *C. rodentium* as well as from isolates of each strain extracted from fecal pellets from infected mice, as has been done by Petty and colleagues. Wild-type *C. rodentium* and Mut1 isolates from various time points should be examined by pulse-field gel electrophoresis (PFGE). Briefly, after serial dilution of fecal pellets and plating onto MacConkey agar plates, single colonies should be picked and grown overnight. Bacterial DNA plugs can be prepared using a CHEF Genomic DNA Plug Kit (Bio-Rad Laboratories) and incubated with a restriction enzyme. The plug is then electrophoresed through an agarose gel, following the manufacturer's directions. This produces a banding pattern that can be compared between the wild-type *C. rodentium* and Mut1 *C. rodentium* from samples pre- and post- infection in mice. Differences in the banding pattern post infection between the wild-type *C. rodentium* and the Mut1 strain would indicate that the interrupted recombinase is functioning to cause changes in the genomic structure of the Mut1 strain.

Following confirmation that the Mut1 strain is defective in recombination, it will be necessary to

identify potential target genes being regulated by the recombinase and how these genes alter virulence. We can identify the potential genes by employing a recently described method based off of paired-end whole genome sequencing (WGS) (Goldberg et al., 2014). In short, genomic DNA extracted from a culture of bacteria should be sheared to generate inserts approximately 500 bp in size. A sequencing library can be generated using the Paired-end Sample Prep Kit (Illumina). This sequencing process provides a short read (90-100 bp) of both ends of an individual insert that can be aligned to the reference genome in order to determine a gap-size (the distance between the paired reads). The genomic location and gap-size for each insert should be plotted resulting in a graph where most of the reads will fall around a gap-size that corresponds to the size to which the genomic DNA was sheared (500 bp). In the event of a genomic inversion, the pair-end sequencing reads will be in the same orientation when aligned to the reference sequence. When reads along an inversion are plotted against the genomic location, their gap-size will be much larger than the rest of the genes and it will form a distinctive pattern on the graph. Using this technique, inversions can be identified along the entire genome. By comparing the paired-end data between the wild-type and Mut1 *C. rodentium* strains we can confirm the activity of the recombinase as well as trace the location of genomic inversions.

The DNA recombinase we identified in this study likely functions in a similar manner as the Salmonella Hin system (Johnson, 2015) or the *Bacteroides fragilis* MPI recombinase (Coyne et al., 2003), which are also serine recombinases. Following the data from the paired-end WGS, we can confirm these inversions by analyzing those genomic locations for serine recombinase consensus motif sequences that should flank genes targeted for inversion by our site-specific recombinase. Inversions at these promoters act as a molecular switch to turn on or off transcription. To determine if these genes are directly regulated by this specific recombinase, the promoters of these genes need to be cloned onto plasmids and tested for inversion using a PCR strategy that produces products when the promoters have undergone inversion but not when they remain unchanged. This is done through the use of the directional primers matching the cloned promoter region and a primer for the plasmid. Upon inversion, the promoter sequence will be in the correct orientation for the two primers to make a product. However when no

inversion occurs, the primers will not be oriented in the appropriate manner for the polymerase to produce a product. For these experiments, it is imperative that the plasmid and our recombinase are transformed into a bacterial host that has no endogenous recombinase activity. This system has been used to study the genes regulated by the MPI serine recombinase in *Bacteroides fragilis* using the bacteria *Bacteroides vulgatus*, which has no endogenous recombinase activity (Coyne et al., 2003). In our case, whether the genes controlled by this site-specific recombinase function to alter the antigenic surface appearance of the bacteria to the immune system or to turn on specific virulence genes needed to invade the host, remains to be seen. Given the little information we have regarding the inflammatory response and the lack of penetration of the epithelial cell layer, it seems reasonable to suggest that this recombinase alters the function of genes involved in gaining access to the epithelial cell layer or regulating the expression of certain key virulence factors, yet further elucidation of the specific targets will required learning more about the limitations of the Mut1 strain, as proposed in the earlier sections.

4.4 Conclusion

While it remains unclear the exact mechanisms causing the lack of virulence associated with the Mut1 strain, there are many avenues of research open for assessment. This approach has provides us with a two pronged approach to gain a deeper understanding of the pathogenesis of *C. rodentium,* through highlighting areas of further research on both the host and the pathogen. Our current studies in the IL-22$^{-/-}$ mice allowed us to interrogate the activity of this mutant *C. rodentium* strain *in vivo*. Through this, we observed that the Mut1 strain's deficiency in directly attaching to the epithelial cells *in vivo* led to a lack of inflammatory response, as measured by real time PCR, and lack of barrier disruption. This attenuated phenotype of Mut1 *C. rodentium* may allow further study of the importance of the IL-22 cytokine during the later phase of the immune response, since the Mut1 strain doesn't induce the rapid mortality associated with wild-type *C. rodentium* infection. Using this host has also raised questions more specifically about the importance of IL-22 during infection with wild-type *C. rodentium* that have yet to be resolved in the literature. Alternatively, the use of the Mut1 strain highlights facets of the *C. rodentium* infection

strategy that are not yet fully understood, such as the mechanism(s) employed to gain access to the epithelial cells. The resolution of the remaining questions may uncover new targets for therapeutics for the treatment and prevention of A/E infections as well as lead to a deeper understanding of the immune response following infection. This information may inform treatment options to boost the immune response and help heal the protect the gut, as this is one of the primary functions ascribed to IL-22 and fuel the further interest in this topic.

4.5 Methods and materials

Animal care and ethics

All animal experiments were approved and performed according to protocol number MO13H349 under the Johns Hopkins University's Animal Care and Use Committee and in direct accordance with the NIH guidelines for housing and care of laboratory animals.

Infection

Male IL-22$^{-/-}$ mice on the C57BL/6 background were kindly shared by Dr. Cynthia Sears (Johns Hopkins University). Mice were maintained in a specific pathogen-free facility and fed autoclaved food and water ad libitum. On infection days, food and water was withheld from the mice for 6–8 hours before they were orally inoculated with 200 µl of PBS containing 2x10^{9} CFU of wild-type or Mut1 *C. rodentium* or PBS alone, and euthanized at the indicated time points post infection as described previously (Hodgson et al., 2015).

Clinical score of colitis

Animals were observed daily for weight and morbidity using the following scoring system (Qualls et al., 2006). Weight loss scores were determined as 0 = no change; 1 = 1%-5% weight loss; 2 = 5%-10% weight loss; 3 = 10%-15% weight loss; 4 = 15% - 20% weight loss; 5 = more than 20% weight loss. Stool consistency scores were determined as 0 = normal, well-formed pellets; 2 = diarrhea, yellowish mud-like

feces; 4 = watery diarrhea. Bleeding was determined as 0 = no bleeding; 2 = slight bleeding; 4 = gross bleeding. The clinical score is the sum of the three parameters, where 0 indicates no clinical symptoms and 13 indicates severe colitis.

Fecal Clearance

Fecal pellets were collected on the indicated days from each animal, weighed, and diluted 0.1 g in 1 ml in sterile PBS. Diluted pellets were vortexed at medium speed and placed on shaker in the cold room for 30 to 60 min. Pellets were vortexed again until fully dissolved. Samples of individual mice were pooled by infection and serially diluted. MacConkey agar plates were used to plate dilutions in triplicate. Plates were incubated overnight at 37°C. Colonies were counted the following day to determine CFU per group.

Systemic Bacterial Dissemination

Spleens and livers were resected from euthanized mice at the indicated time points and were weighed and homogenized. Homogenates were serially diluted and plated in triplicate on MacConkey agar. *C. rodentium* CFUs were measured as described above.

Histology (H/E and IFA)

Following euthanasia, the colons of mice were harvested under aseptic conditions, washed once with ice-cold PBS, and the terminal 0.5-cm section of the colon was either frozen in optimal cutting temperature (O.C.T.) media (Tissue-Tek, Elkhart, IN) or incubated overnight in 4% PFA. 5-micron frozen sections were cut using a Microm HM 550 Cryostat (Thermo Scientific), collected on coated slides and processed for immunofluorescence staining. Frozen sections were fixed in 4% PFA, washed with PBS, and blocked with appropriate sera in PBS. After incubating with appropriate antibodies, sections were washed and incubated with fluorescence dye-conjugated second antibodies and 1 μg/ml of DAPI (Sigma-Aldrich). Stained sections were washed and mounted under a coverslip using Fluoro-gel with Tris Buffer (Electron Microscopy Sciences).

For histological analysis, the colon tissue was paraffin embedded and 5-micron sections were cut, collected on coated slides and processed for Hematoxylin and Eosin (H&E) staining. Stained sections were examined using an Axio Observer fluorescence microscope (Zeiss). Histopathology scores were determined in a blinded fashion using the following criteria as previously described (Qualls et al., 2006): 0, Normal tissue; Grade 1, mild inflammation was present containing mostly mononuclear cell infiltrate and little damage to the epithelia; Grade 2, inflammation greater than Grade 1 with mononuclear and polymorphonuclear infiltrate, mucin and Goblet cell depletion, and epithelium beginning to detach from basement membrane; Grade 3, inflammation and cellular infiltrate is greater than Grade 2 with cellular infiltrates reaching the submucosa, greater Goblet cell depletion, and greater epithelial disruption; Grade 4, severe inflammation containing mostly neutrophils, completely detached epithelium, and crypt destruction.

In vitro attachment

Colon epithelial cells (CECs) were isolated from C57BL/6J mice as previously described (Flint et al., 1991). Briefly the colon was resected under aseptic conditions and washed twice with ice-cold PBS. After opening the colon longitudinally and then dividing the colon into 2–3 mm long fragments, the tissue pieces were transferred into chelating buffer (27 mM trisodium cirtcrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 0.5 mM DTT, 55 mM D-sorbitol, 44 mM sucrose, 6 mM EDTA, 5 mM EGTA [pH 7.3]) for 25 min at 4°C. CECs were then further dislodged by repeated vigorous shaking. Tissue debris was removed by a 70-μm cell strainer (Fisher Scientific, Suwanee, GA) and CECs were harvested by centrifugation at 4°C. CEC viability was confirmed by trypan blue staining and isolated CECs were cultured at 37°C for 1 h for recovery, followed by infection with *C. rodentium* at MOI 100 in antibiotic free media. Post *C. rodentium* infection, infected CECs were spun down to Poly-L-Lysine-coated coverslips, fixed with 4% PFA, and stained with appropriate primary antibodies and fluorescence dye-conjugated second antibodies. Following staining of nuclei with 1 μg/ml of DAPI (Sigma-Aldrich), coverslips were mounted onto slides using Fluorogel with Tris Buffer (Electron Microscopy Sciences,

Hatfield, PA) and examined using an Axio Observer fluorescence microscope (Zeiss, Oberkochen, Germany). The numbers of *C. rodentium* that attached to mouse CECs were quantified using ImageJ software (NIH, Bethesda, MD) and normalized to cell perimeter.

RT-PCR and primers

Total RNA was isolated from colon tissues at the indicated time points using Trizol reagent (Life Technologies) and treated with the TURBO DNA-free Kit (Life Technologies) to remove residual genomic DNA. cDNA was synthesized using qScript cDNA SuperMix Kit (Quanta Biosciences, Gaithersburg, MD) according to the manufacturer's instructions. Gene specific products were amplified using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) with the following primers:

Cxcl1-f, 5'-TGCACCCAAACCGAAGTCAT-3';

Cxcl1-r, 5'-TTGTCAGAAGCCAGCGTTCAC-3';

Cxcl2-f, 5'-CCTGCCAAGGGTTGACTTCA-3';

Cxcl2-r, 5'- TTCTGTCTGGGCGCAGTG-3';

Ifng-f, 5'-ATGAACGCTACACACTGCATC-3';

Ifng-r, 5'- CCATCCTTTTGCCAGTTCCTC-3';

Cxcl5-f, 5'-TCCAGCTCGCCATTCATGC-3';

Cxcl5-r, 5'-TTGCGGCTATGACTGAGGAAG-3';

Nos2-f, 5'-GCCACCAACAATGGCAACA -3';

Nos2-r, 5'- CGTACCGGATGAGCTGTGAA-3';

Il6-f, 5'-CCAAGAGGTGAGTGCTTCCC-3';

Il6-r, 5'-CTGTTGTTCAGACTCTCTCCCT-3';

Il17a-f, 5'- TTTAACTCCCTTGGCGCAAAA -3';

Il17a-r, 5'- CTTTCCCTCCGCATTGACAC-3';

Il22bp-f, 5'- TGTGACCTGACCAATGAAACC -3';

Il22bp-r, 5'- CGACCGGAGGATCTAGTTTTGT-3';

Mmp9-f, 5'- CTGGACAGCCAGACACTAAAG -3';

Mmp9-r, 5'- CTCGCGGCAAGTCTTCAGAG-3';

Mmp10-f, 5'-GAGCCACTAGCCATCCTGG -3';

Mmp10-r, 5'- CTGAGCAAGATCCATGCTTGG-3';

Mmp13-f, 5'-TGTTTGCAGAGCACTACTTGAA -3';

Mmp13-r, 5'-CAGTCACCTCTAAGCCAAAGAAA -3';

Icam1-f, 5'- GTGATGCTCAGGTATCCATCCA-3';

Icam1-r, 5'- CACAGTTCTCAAAGCACAGCG-3';

Il2-f, 5'-CCTGAGCAGGATGGAGAATTACA -3';

Il2-r, 5'- TCCAGAACATGCCGCAGAG-3';

Il4-f, 5'- GGTCTCAACCCCCAGCTAGT -3';

Il4-r, 5'- GCCGATGATCTCTCTCAAGTGAT -3';

Il10-f, 5'- GCTCTTACTGACTGGCATGAG-3';

Il10-r, 5'- CGCAGCTCTAGGAGCATGTG-3';

Il12-f, 5'- AGTTTGGCCAGGGTCATTCC-3';

Il12-r, 5'-TCTCTGGCCGTCTTCACCAT -3';

Il20-f, 5'- TCTTGCCTTTGGACTGTTCTCC-3';

Il20-r, 5'- GTTTGCAGTAATCACACAGCTTC-3';

Il21-f, 5'-GGACCCTTGTCTGTCTGGTAG -3';

Il21-r, 5'- TGTGGAGCTGATAGAAGTTCAGG-3';

Il23-f, 5'-TGTTGCCCTGGGTCACTCA -3';

Il23-r, 5'- CCAGGCTAGCATGCAGAGATT-3';

Vcam1-f, 5'-GTGACTCCATGGCCCTCACT -3';

Vcam1-r, 5'- CGTCCTCACCTTCGCCTTTA-3';

PCR for mutation identification

Bacterial cultures were grown overnight, 1ml of fresh culture was pelleted, washed in dH_2O twice, and resuspended in 1ml dH_2O. 5µl of bacteria was used a template for round 1 of PCR. Forward PCR primers were designed containing an artificial tag, a random sequence spacer, and an anchor sequence that appeared in the genome at approximately 250 bp intervals. The reverse primer was specific to the

known transposon sequence. These primers used for PCR with the MyTaq Red Mix (BIOLINE, Massachusetts). The PCR conditions were as follows: 95°C for 5 min; 95°C for 30 sec, 42°C for 30 sec, 72°C for 3 minutes (cycle 6 times); 95°C for 30 sec, 52°C for 30 sec, 72°C for 3 minutes (cycle 25 times). Dilute final product in 80µl of H_2O and use 5µl for round 2 of PCR. A forward primer for the artificial tag from round 1 and a reverse primer for another transposon sequence were used for round 2. PCR conditions were as follows: 95°C for 5 min; 95°C for 30 sec, 45°C for 30 sec, 72°C for 3 minutes (cycle 40 times). PCR clean up (Macherey-Nagel, Duren, Germany) was preformed according to manufacturer's directions and samples were sent for sequencing. Sequencing results were screened for gene matches using BLAST (NCBI). Primers were generated specific to the identified gene for confirmation.

Forward round 1- 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNCGGCG-3'

Reverse round 1- 5'-CGTTAAAACTGCCTGGCACAGCA-3'

Forward round 2- 5'-GGCCACGCGTCGACTAGTAC-3'

Reverse round 2- 5'-CGTCGAGTTTTTTTGATTTCACGGG-3'

Statistics

All statistical analysis was performed using GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). The difference between treated and control groups were examined by unpaired Student's t tests. Standard errors of means (s.e.m.) were plotted in graphs. n.s. means non-significant difference and significant differences were considered 0 at $p < 0.05$; 00 at $p < 0.01$; and 000 at $p < 0.001$.

4.6 Figures

Figure 4.1 Mut1 causes limited morbidity and mortality in IL-22$^{-/-}$ mice.

Mice were infected with either wild-type *C. rodentium* or Mut1 *C. rodentium* and monitored for weight loss (A), clinical score (B), and survival (C). Clinical score was measured as the sum of colitis disease scores for weight loss (1-5 points) and stool consistency (0-4 points).

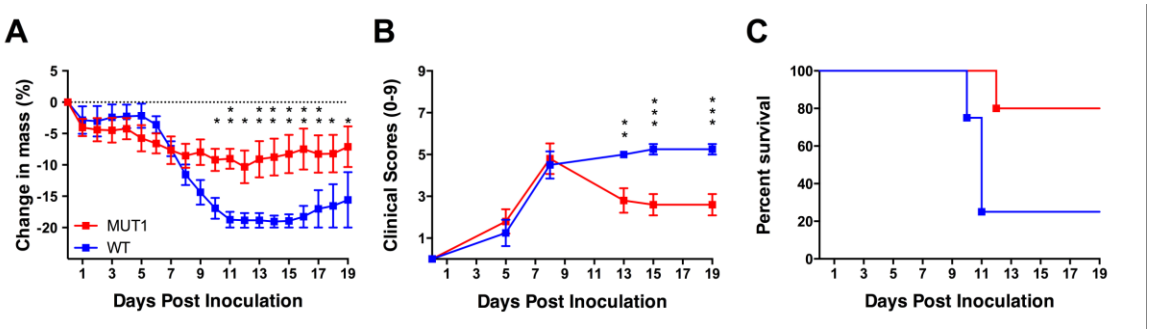


Figure 4.2 Mut1 *C. rodentium* has no growth or attachment defects.

(A) OD readings were taken every hour for Mut1 *C. rodentium* and wild-type *C. rodentium* strains that were equally diluted in LB broth. OD600 readings were plotted by time. (B) CECs were harvested from IL-22$^{-/-}$ mice and infected with either Mut1 *C. rodentium* or wild-type *C. rodentium in vitro*, fixed, and stained as indicated. The number of adherent bacteria were counted per cell in 20 random fields and normalized to cell size. Scale bar, 10 um. (C) The fecal load of Mut1 *C. rodentium* and wild-type *C. rodentium* was determined by serial dilution of fecal pellets collected from IL-22$^{-/-}$ mice at the indicated time points. (D) The colons of Mut1 *C. rodentium* and wild-type *C. rodentium* infected IL-22$^{-/-}$ mice were isolated, fixed, and paraffin embedded at day 8 post infection. Tissue sections were stained as indicated to determine bacterial adherence. Scale bars, 50um.

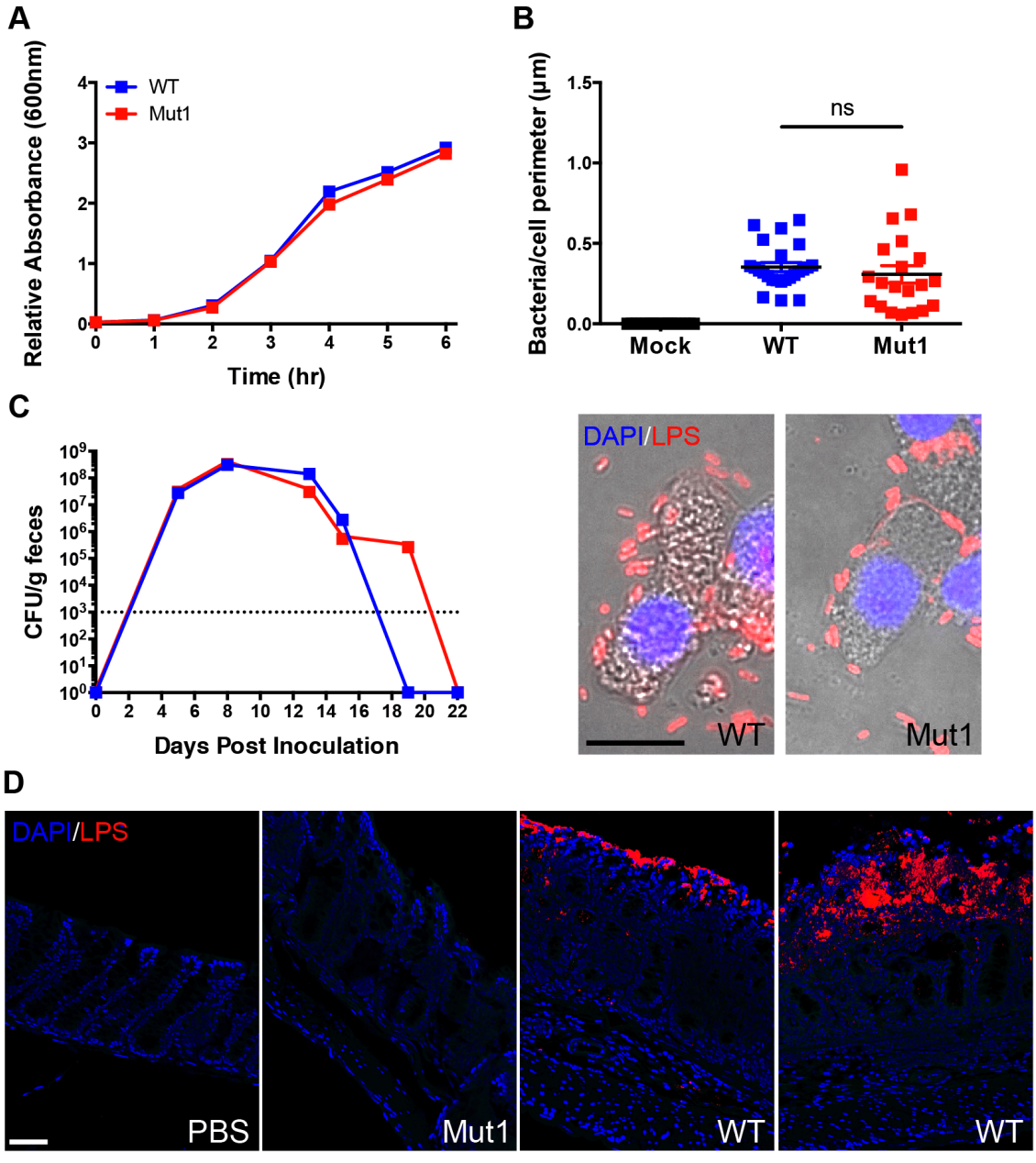

A
Relative Absorbance (600nm)
WT
Mut1
Time (hr)
B
Bacteria/cell perimeter (µm)
ns
Mock
WT
Mut1
C
CFU/g feces
Days Post Inoculation
DAPI/LPS
WT
Mut1
D
DAPI/LPS
PBS
Mut1
WT
WT

Figure 4.3 Mut1 infection induces limited intestinal inflammation.

IL-22$^{-/-}$ mice were infected with PBS, Mut1 *C. rodentium*, or wild-type *C. rodentium* and colon tissue was harvested at day 8-post infection. (A) Colon tissue was paraffin embedded and H&E staining was performed to examine the tissue pathology. Scale bar, 100 um. (B) RNA was extracted from 1cm long colon tissue sections. RT-PCTR analysis was done for the indicated genes.

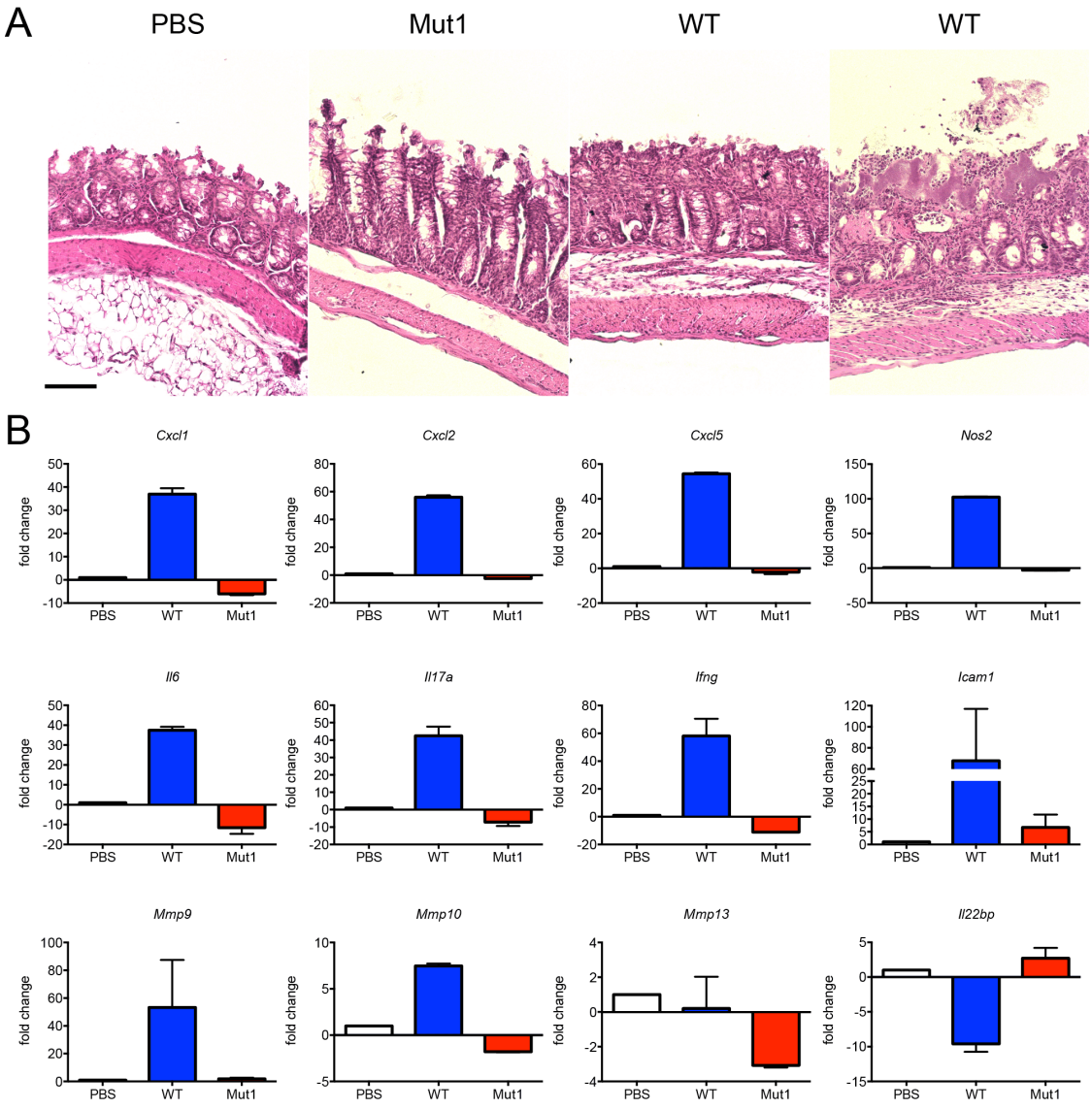


Figure 4.4 Continued RT-PCR panel.

IL-22$^{-/-}$ mice were infected with PBS, Mut1 *C. rodentium*, or wild-type *C. rodentium* and colon tissue was harvested at day 8-post infection. RNA was extracted from 1cm long colon tissue sections. RT-PCTR analysis was done for the indicated genes.

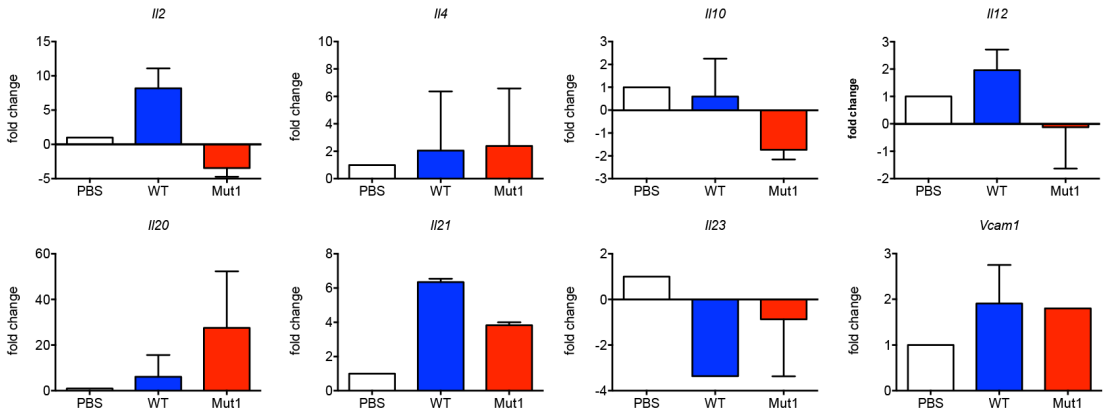


Figure 4.5 Mut1 infection does not alter barrier permeability.

IL-22 KO mice infected with PBS, Mut1 *C. rodentium*, or wild-type *C. rodentium* were euthanized at day 8-post infection. (A) Colon tissue was paraffin embedded and stained with anti-claudin-3. Scale bar, 20 um. (B) The liver and spleen of each animal was resected, homogenized in PBS, and plated onto MacConkey agar plates to determine the presence of bacteria in these tissues.

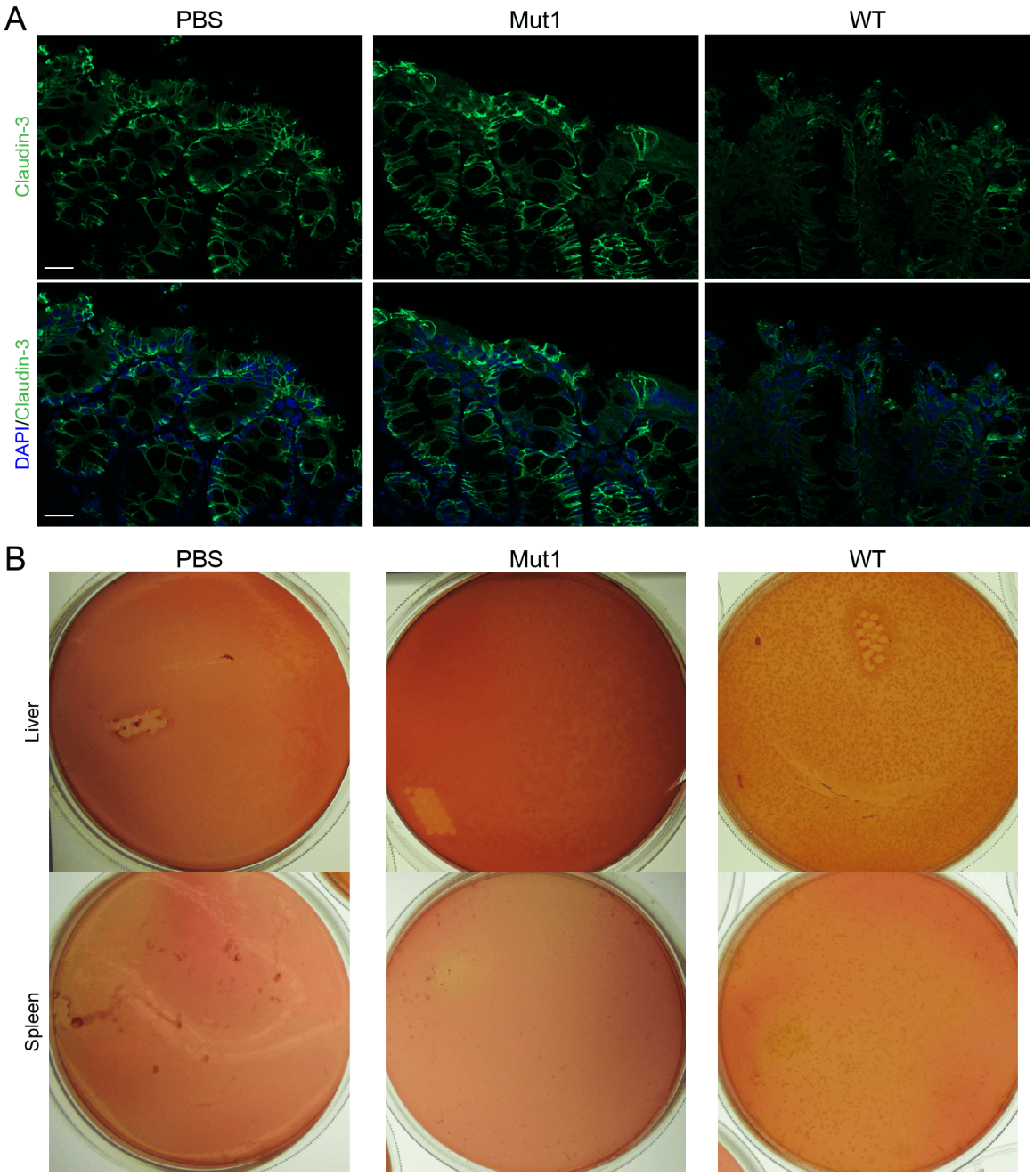
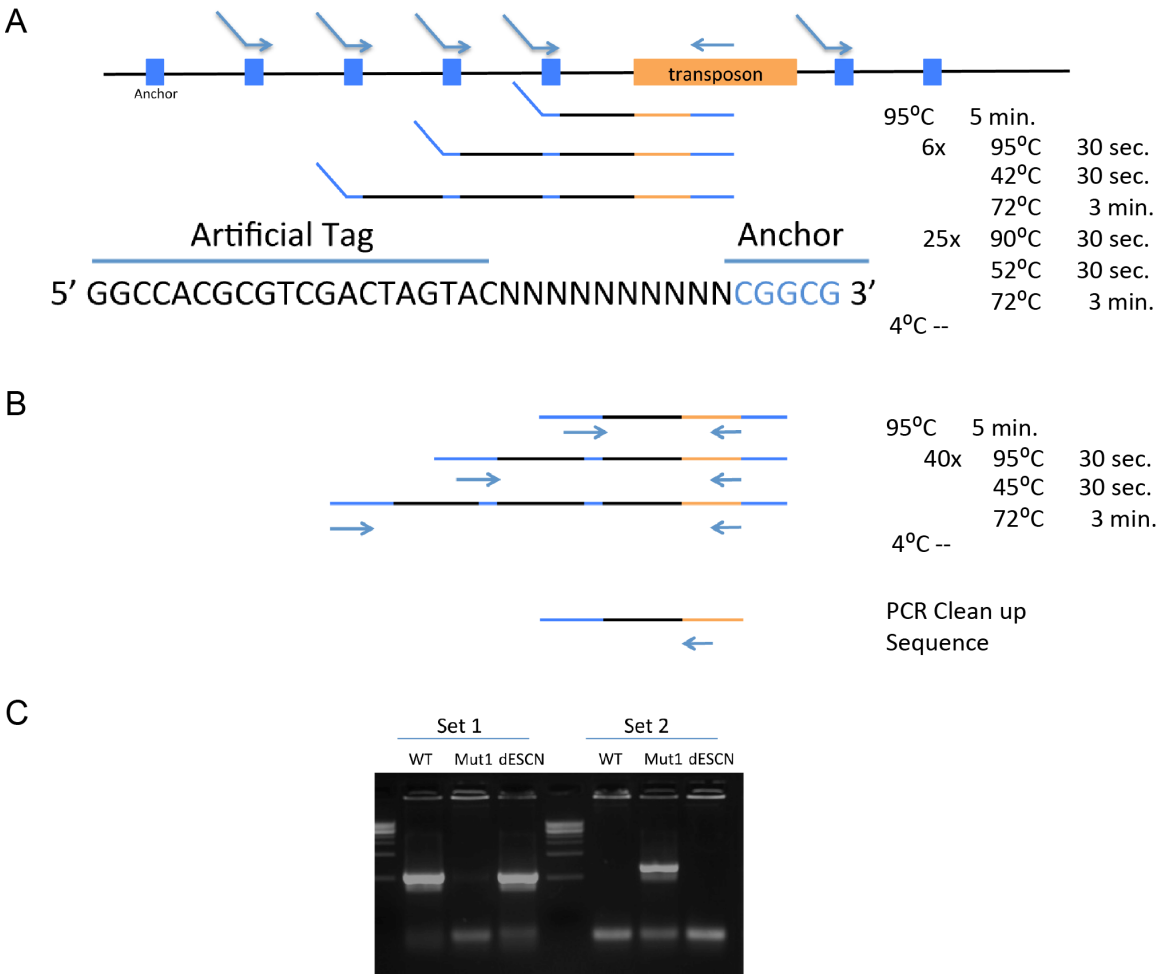


Figure 4.6 Mut1 *C. rodentium* carries a disruption in a site-specific recombinase gene.

Schematic of the round 1 (A) and round 2 (B) PCR primers and procedure used to generate the DNA fragment sent to sequencing to identify the area of disruption. (C) DNA gel electrophoresis of the products obtained from a PCR screen done to confirm gene.



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5 Conclusion

The constant battle between invading pathogens and the host immune system is a fascinating and ever evolving area of study. Gaining a deeper understanding of the mechanisms involved in both sides of the equation, would allow us to take more informed approaches toward the development of therapeutics and preventative measures. However, there are many challenges in studying host pathogen interactions *in vivo* and we are therefore forced to rely on reductionist and *in vitro* approaches. These approaches are very powerful in elucidating the detailed interactions that occur between proteins, but they must be brought back to the *in vivo* level for confirmation and validation. More specifically, three major hurdles that would greatly advance the study of how pathogen-encoded proteases alter host signaling would be 1) *in vivo* or in infection verification of substrate specificity to avoid pseudo-positive targets identified by *in vitro* protease assays; 2) a unified system to measure protease activity that would allow for better control and comparison of studies from different research groups; and 3) more sensitive detection methods to study the spatial-temporal activity, localization, and induction of pathogen-encoded proteases (or other virulence factors) during pathogen infections.

Arguably, the development of more sensitive strategies for the detection of low expression level virulence factors would provide the greatest advantage to the field. The timing of when effectors are active during infection may be key to understanding why some studies report gene expression activation and others can report repression for the same pathogen. During infection by *Citrobacter*, Leishmania, and poliovirus (as discussed in chapters 2 and 3) the exact timing of the protease expression and cleavage events would clarify the delicate balance between activating NF-κB signaling and selectively turning off part of the response. While strong antibodies exist for the poliovirus 3C protease and at later stages of infection it is expressed at high levels, it is likely that early events are going undetected before the expression of 3C is greatly amplified during the viral infection cycle. Similarly, NleC is introduced into cells at low levels and determining its function and localization is difficult to do using infection conditions. It is also difficult to extrapolate what concentration of the protease would be appropriate for

in vitro experiments to delineate a pathophysiological relevant response. Improvements in these areas would generally benefit the study of host-pathogen interactions and protease activity and regulation. Pathogens have evolved multiple effective strategies to counteract the host immune response to infection and understanding the intricacies of these interactions will reveal more about the host immune response that had previously gone unnoticed. Our model (chapter 2) demonstrates that as the mechanisms of NF-κB promoter specificity are further elucidated, studies demonstrating cleavage events of NF-κB proteins need to further characterize the fate of the cleaved products to determine if they have a biological function in the context of infection or host-mediated cleavage.

The effect that complex host-pathogen protein interactions have on the global response to infection requires further inquiry. Basic research has enabled us to gain in-depth knowledge about the regulation of very specific protein functions and their effect on transcription. However, as technology advances, it allows for the greater examination of the crosstalk between multiple signaling pathways; thus it is becoming more important to holistically incorporate these functions into disease models. For example, as more "specifier" type proteins are uncovered to dictate the promoter selectivity of NF-κB signaling it will become important to define the specific target genes that are regulated by each "specifier" protein. These cohorts of genes can be identified through the use of knockdowns and chromatin-immunoprecipitation combined with sequencing (ChIP-seq). Following these advancements, profiles of NF-κB activation can be constructed relating to the specific stimuli and "specifier" function. Such frameworks, akin to the CD4 T cell development profiles, will enable processing of the implications when a particular "specifier" is compromised, either through genetic mutation or pathogen interference.

In the context of our current work (chapter 2), the implication of selectively blocking RPS3-dependent genes is that the tempered immune response provides an advantage to the pathogen. It is known that following *C. rodentium* infection, IL-22 is induced and we have demonstrated an increase in IL-22 expression when NleC is unable to block RPS3. IL-22 is critical for protecting the host from *C. rodentium* induced pathology and mortality, therefore regulating the expression of IL-22, via RPS3-

blockade, likely benefits the bacteria. In this way *C. rodentium* can allow for low level IL-22 production, without inducing robust expression of mucins and anti-microbial peptides. Moreover, by selectively blocking RPS3-dependent genes but not global NF-κB-mediated transcription *C. rodentium* is modulating its environment to better suit its needs. Follow up studies should serve to link the host-pathogen protein-protein interactions we observed to the larger tissue protective effects the induced immune response attempts to produce.

It remains to be seen whether the selective regulation of NF-κB has an impact on the fitness of *C. rodentium*, independent of the effect on the host. In this regard, understanding how the inflammatory environment of the infected host alters bacterial gene expression remains to be elucidated. It is currently understood that *C. rodentium* induces the expression of certain virulence operons following exposure to bicarbonate within the gut, demonstrating that virulence genes can be regulated by environmental exposures. The use of immunocompromised hosts, such as the IL-22$^{-/-}$ mouse, may be key to further revealing the regulatory mechanisms of bacterial virulence gene transcription as host defects may reveal signals that induce bacterial gene expression. Alternatively, using mutant bacterial strains such as our attenuated Mut1 *C. rodentium* strain that seemingly lack a DNA recombinase can also serve to identify novel regulatory mechanisms of bacterial gene expression. Given the inability to induce robust inflammation even in a compromised host suggests that the genes controlled by the DNA recombinase play a vital function in pathogenesis. However, as discussed in chapter 4, a significant amount of work is still required to understand the attenuation of the Mut1 strain and characterize the DNA recombinase protein given that we do not yet know which genes are regulated by this recombinase.

The complex environment of the gastro-intestinal tract poses serious challenges for studying the *in vivo* interactions involved. Yet through a combination of *in vitro* studies, the development and use of new infection model systems and permissive hosts, and a better understanding of pathogen gene regulation significant contributions can be made to the field. Our studies discussed here serve as an example of how novel regulatory mechanisms at the protein level should be further examined to evaluate their role in the

context of infection. By examining the regulatory mechanisms of gene expression and how either side hijacks these mechanisms will undoubtedly serve to further our understanding of the complex host pathogen interactions we are subject to during infection.

6 CV

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EDUCATION

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Ph.D., Molecular Microbiology and Immunology 2015
Advisor: Dr. Fengyi Wan
Dissertation: Interference mechanisms of attaching/effacing pathogens with the host inflammatory response (abstract attached)
Certificates: Clinical Vaccine Trials and Good Clinical Practice 2015
Vaccine Science and Policy 2015

University of Rhode Island, Kingston, RI
B.S., Microbiology, *Magna Cum Laude* 2008

RESEARCH EXPERIENCE

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD July 2012-Sept. 2015
Graduate Research Assistant
Advisor: Dr. Fengyi Wan

- Examined the molecular mechanisms through which pathogenic *Escherichia coli* interfere with host NF-κB signaling pathway and the inflammatory response
- Initiated projects examining the role of IL-22 in the pathogenesis of *Citrobacter rodentium* infection; infection and inflammation related colon cancer
- Initiated and developed a project examining the behavioral characterization of a knockout mouse strain
- Responsible for the training of all graduate students in animal work, particularly infection (BSL-2) based projects

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD Sept. 2009-July 2012
Graduate Research Assistant
Advisor: Dr. Sabra L. Klein

- Examined sex differences in the immune response to influenza infection and cross protection
- Initiated project to determine the effect of estradiol on the pathogenesis of influenza A virus on primary respiratory epithelial cells

Clean Harbors/ Bristol-Myers Squibb, Princeton, NJ Jan. 2009-June 2009
Media lab technician

- Duties included managing and operating the media laboratory, preparing solutions, delivering reagents to labs, autoclaving, etc.

University of Rhode Island, Kingston, RI Jan. 2008-Dec. 2008
Undergraduate Research Assistant
Advisor: Dr. Matthew Stoner

- Characterized novel compounds for their anti-estrogenic effect on gene expression in hormone responsive breast cancer cell lines

University of Rhode Island, Kingston, RI Jan. 2006-Dec. 2007
Undergraduate Research Assistant
Advisor: Dr. Albert P. Kausch

- Duties included developing and expanding a tissue culture system for switchgrass, transfection and transformation, training high school students

PEER-REVIEWED PUBLICATIONS

Hodgson, A. and Wan, F. (2015), Interference with NF-κB signaling pathway by pathogen-encoded proteases: global and selective inhibition. Molecular Microbiology. Accepted Author Manuscript. doi:10.1111/mmi.13245

Hodgson, A*., Wier, E.M*., Fu, K., Sun, X., Yu, H., Zheng, W., Sham, H.P., Johnson, K., Bailey, S., Vallance, B.A*., et al.* (2015). Metalloprotease NleC suppresses host NF-kappaB/inflammatory responses by cleaving p65 and interfering with the p65/RPS3 interaction. PLoS pathogens *11*, e1004705. PMID: 25756944. (*Co-first author)

Lasola, J*., Hodgson, A*., Sun, X*., and Wan, F. (2014). The PARP1/ARTD1-Mediated Poly-ADP-Ribosylation and DNA Damage Repair in B Cell Diversification. Antibodies *3*, 37–55. (*Co-first author)

Fu, K., Sun, X., Zheng, W., Wier, E.M., Hodgson, A., Tran, D.Q., Richard, S., and Wan, F. (2013). Sam68 modulates the promoter specificity of NF-kappaB and mediates expression of CD25 in activated T cells. Nature communications *4*, 1909. PMID: 23715268.

Klein, S.L., Hodgson, A., and Robinson, D.P. (2012). Mechanisms of sex disparities in influenza pathogenesis. Journal of leukocyte biology *92*, 67-73. PMID: 22131346.

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POSTER PRESENTATIONS AND PUBLISHED ABSTRACTS

Hodgson, A., Wier, E.M., Fu, K., Sun, X., Yu, H., Zheng, W., Sham, H.P., Johnson, K., Bailey, S., Vallance, B.A., Wan, F. 2015. Selective regulation of NF-κB by cleavage of p65 and interfering with the p65/RPS3 interaction by A/E pathogen. American Association of Immunity, New Orleans, LA.

Hodgson, A., Wier, E.M., Fu, K., Sun, X., Yu, H., Zheng, W., Sham, H.P., Johnson, K., Bailey, S., Vallance, B.A., Wan, F. 2014. Selective regulation of NF-κB by cleavage of p65 and interfering with the p65/RPS3 interaction by A/E pathogen. Biochemistry and Molecular Biology Annual Retreat, Baltimore, MD.

Hodgson, A., Lalime, E. N., Pekosz, A., & Klein, S. L. 2012. Estrogen Receptor Expression and Effects of Estradiol on Murine Tracheal Epithelial Cell Function. Organization for the Study of Sex Differences, Baltimore, MD.

Hodgson, A., Lalime, E. N., Pekosz, A., & Klein, S. L. 2011. Estrogen Receptor Expression and Effects of Estradiol on Murine Tracheal Epithelial Cell Function. Molecular Microbiology and Immunology Departmental Retreat, Hershey, PA.

Hodgson, A., Lorenzo, M. E., Robinson D. P., Pekosz, A., & Klein, S. L. 2011. Adaptive Immunity and Cross Protection Against Influenza Virus Infection Differs Between the Sexes. Organization for the Study of Sex Differences, Oklahoma City, OK.

Hodgson, A., Lorenzo, M. E., Robinson D. P., Pekosz, A., & Klein, S. L. 2011. Sex Differences in Adaptive Immunity and Cross Protection to Influenza A Viruses. Women's Health Research Group at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

Lorenzo, M. E., **Hodgson, A.,** Pekosz, A., & Klein, S. L. 2010. Sex differences in cross protective immunity against lethal influenza A viruses. Cell Symposium Influenza: Translating Basic Insights, Washington, DC.

Lorenzo, M. E., **Hodgson, A.,** Pekosz, A., & Klein, S. L. 2010. Cross protective immunity against lethal influenza A viruses is greater in females than males. 2010 Vaccine Day at Johns Hopkins Bloomberg School of Public Health, Baltimore, MD.

Lorenzo, M. E., **Hodgson, A.,** Pekosz, A., & Klein, S. L. 2010. Cross protective immunity against lethal influenza A viruses is greater in females than males. Molecular Microbiology and Immunology Departmental Retreat, Hershey, PA.

Lorenzo, M. E., **Hodgson, A.,** Pekosz, A., & Klein, S. L. 2010. Cross protective immunity against lethal influenza A viruses is greater in females than males. 20th Anniversary of the Office of Women's Health Research Symposium, Bethesda, MD.

Hodgson, A., Hamel, L.D., Anderson, L., Couto, E., Williams Jr., J.C., Stoner, M.A. 2008. Synthesis and *in vitro* screening of novel anti-estrogens. 3rd Annual Biology New England South Meeting, Bristol, RI

Hodgson, A., Hamel, L.D., Anderson, L., Couto, E., Williams Jr., J.C., Stoner, M.A. 2008. Synthesis and *in vitro* screening of novel anti-estrogens. Northeast Chapter of the Society of Toxicology Fall Meeting, Shrewsbury, MA.

HONORS AND AWARDS

2005-2008	Centennial Scholarship
2005-2008	Phi Eta Sigma National Honor Society
2008	Louis Stokes Alliances for Minority Participation Scholar
2008	Summer Undergraduate Research Fellowship Rhode Island –IDeA Network of Biomedical Research Excellence
2008	Norman and Alicia Tashash Scholarship
2008	Golden Key International Honor Society

2008	Biology New England South Meeting Student Poster Award
2009	Society of Toxicology Minority Undergraduate Education Program Travel Award
2010- 2011	Frances A. Coventry Fund recipient
2011-2015	Johns Hopkins Sommer Scholar
2015	Delta Omega Poster Competition Award
2015	Federation of American Societies for Experimental Biology MARC Travel Award- AAI

ASSOCIATION MEMBERSHIPS

The American Association of Immunologists (AAI); American Society for Microbiology (ASM)

TECHNICAL SKILLS

Molecular Biology

- Construct design via traditional molecular cloning, In-Fusion cloning, and site-directed mutagenesis
- Protein expression in mammalian cell culture via transfection
- Genomic DNA and RNA extraction
- Expression profiling: qPCR, western blotting, ELISA, flow cytometry, immunoprecipitation, luciferase assays
- Immunohistochemistry of cell culture cells and tissue samples (fixation to imaging)
- Fluorescence microscopy via Axio Observer Microscope (Ziess)
- Mammalian cell culture: standard and air-liquid interphase (ALI) cultures, cell lines and primary cells, subcellular fractionation
- Bacteriology: culture, serial dilution, *in vitro* and *in vivo* infections, BSL-2 training
- Virology: TCID50, reverse genetics system, viral neutralization assays, *in vitro* and *in vivo* infections, BSL-2 training
- Rodent animals skills (mice): breeding, tagging, genotyping, injection (IM and IP), oral gavage, intranasal infection, dissection, retro-orbital bleeds, antibody titers, DSS colon cancer model, Min^{APC} model, forced swim test

Software

- Microsoft Office (Word, Excel, Outlook, Powerpoint)
- Graphpad Prism
- Imaging software: ImageJ, Zen Pro, Alphaview

LANGUAGE SKILLS

- English
- Spanish

MENTORING EXPERIENCE

Graduate Students

Yue "Harry" Liu (2014-2015)
Summer Xia (2014-2015)
Christine Lee (2013)
Grace Hwang (2013)

Undergraduate Students
Shantini Persaud (2011-2012)
Phil Mac (2014-2015)

High School Students
Shaquetta Walker (2013)

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